

STEM CELL BASED GENE THERAPY FOR POMPE'S DISEASE

MEREL STOK

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Stem cell based gene therapy for Pompe's disease

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STEM CELL BASED GENE THERAPY FOR POMPE'S DISEASE

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PROMOTIECOMMISSIE

Promotoren:

Prof.dr. G. Wagemaker

Prof.dr. A.G. Vulto

Overige leden:

Prof.dr. P.A.E. Sillevs Smitt

Prof.dr. A.T. van der Ploeg

Prof.dr. F. Mavilio

Copromotoren:

Dr. A.J.J. Reuser

Dr. N.P. van Til

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Stem cell based gene therapy

for Pompe's disease

acid alpha-glucosidase

Integrations

Hematopoietic stem cells

Muscle

Mouse

Single intervention

Lentivirus

Future

Storage

ERT

Transplantation

Costs

Glycogen

Immune tolerance

Transplantation

GENERAL INTRODUCTION LYSOSOMAL STORAGE DISORDERS

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According to the European Organization for Rare Disease (EURORDIS), about 30 million people in Europe are affected by one of the 6,000-8,000 rare diseases that are currently known¹. The number of people affected in the European Union amounts to 6-8% of the total population. Most rare diseases (80%) are genetically determined, and a small percentage of those represent “lysosomal storage disorders (LSDs)”^{2,3}. They are caused by protein deficiencies that affect the function of an intracellular organelle called “lysosome”. Most LSDs are monogenic disorders that result from a single genetic defect leading to the absence or dysfunction of a single protein -usually an enzyme- that can, however, interfere with more than one metabolic or catabolic pathway. This group of LSDs consists of approximately 50 different metabolic diseases and displays a broad variety of disease manifestations depending on the functional (enzyme) deficiency, the particular pathway involved, and the kind of resulting lysosomal storage products. The overall incidence of LSDs is approximately 1: 5,000, however, the incidence of individual diseases is much more rare. For example the incidence of Pompe’s disease and Fabry’s disease is 1:40,000. The pattern of inheritance of almost all LSDs is autosomal recessive. A few of them are X-linked inherited⁴.

With their content of approximately 50 lysosomal enzymes (mostly hydrolases) lysosomes are able to degrade virtually every cellular substance and play a crucial role in cellular renewal via recycling. The products to be recycled enter the lysosomes through processes of (micro) autophagy, and endocytosis⁵.

LSDs are progressive diseases, and treatment should start before irreversible tissue damage has occurred. However, for the majority of LSDs, there is no effective therapy available, and supportive care (SuC) is the only option (Table 1). Depending on the disease-associated symptomatology the support is very diverse and can include, for instance, a wheelchair or assisted ventilation, while surgical interventions, physiotherapy and dietary advices are also applied⁶. For some of the LSDs enzyme replacement therapy (ERT) is a treatment option. Patients on ERT receive every week or every second week an intravenous administration of the deficient enzyme to temporarily clear the products that have accumulated in the lysosomes. Specifics about the application of ERT will be discussed later in this chapter.

Another treatment option in a small number of other LSDs is bone marrow transplantation. For example in patients with mucopolysaccharidosis type I (MPSI) a bone marrow transplant (BMT) can improve disease symptoms⁷.

As shown in Table 1, there is no permanent cure for any of the LSDs, emphasizing the need for innovative therapeutic developments. One of the promising approaches for LSDs is gene therapy. The basis for gene therapy in LSDs is the common assumption that these diseases arise from the inheritance of mutated genes. When one is able to substitute or re-

Table 1: Overview of LSDs and available treatments at the time of writing this manuscript. SuC; supportive care, ERT, enzyme replacement therapy; HSCT, hematopoietic stem cell transplantation; GT*, ongoing gene therapy clinical trial (source: <http://ghr.nlm.nih.gov> and clinicaltrials.gov).

Storage disorder	Defective enzyme	Treatment options
α -Mannosidosis	α -Mannosidase	SuC, HSCT
β -Mannosidosis	β -Mannosidase	SuC
Cystinosis	Cystine transport protein	SuC, cysteamine
Danon	LAMP-1	SuC
Fabry	α -Galactosidase	SuC, ERT, HSCT, GT*
Fucosidosis	α -L-Fucosidase	SuC, HSCT
Galactosialidosis	Protective protein	SuC, GT
Gaucher	Glucocerebrosidase	SuC, ERT, HSCT, GT*
GM1 gangliosidosis, MPS IVB	Acid β -galactosidase	SuC, ERT, HSCT
GM2 gangliosidosis, AB variant	GM2 activator protein	SuC
GM2 gangliosidosis, Sandhoff	Hexosaminidase A&B	SuC
GM2 gangliosidosis, Tay-Sachs	Hexosaminidase A	SuC, HSCT
Krabbe	Galactocerebrosidase	SuC, HSCT
Metachromatic leukodystrophy	Arylsulfatase A	SuC, HSCT, GT*
Mucopolidosis I, Sialidosis	Sialidase	SuC
Mucopolidosis II, I-cell disease & III, pseudo-Hurlerpolydystrophy	UDP-N-Ac-glucosaminyl phosphotransferase	SuC, HSCT
Mucopolidosis IV	Mucolin I	SuC
Mucopolisaccharidosis I, Scheie/Hurler-Scheie/Hurler	α -L-Iduronidase	SuC, ERT, HSCT, GT
Mucopolisaccharidosis II, Hunter	Iduronate-2-sulfatase	SuC, ERT, HSCT, GT*
Mucopolisaccharidosis IIIA, Sanfilippo	Glucosamine-N-sulfatase	SuC, HSCT, GT*
Mucopolisaccharidosis IIIB, Sanfilippo	α -N-Ac-glucosaminide-N-acetyltransferase	SuC
Mucopolisaccharidosis IIIC, Sanfilippo	AcCoA: α -glucosaminide-N-acetyltransferase	SuC
Mucopolisaccharidosis IIID, Sanfilippo	N-acetylglucosaminide-6-sulfatase	SuC
Mucopolisaccharidosis IVA/B, Morquio A/B	N-acetylgalactosamine-6-sulfatase / beta-galactosidase	SuC, ERT, GT*
Mucopolisaccharidosis VI, Maroteaux-Lamy	Arylsulfatase B	SuC, ERT, HSCT, GT*
Mucopolisaccharidosis VII	β -Glucuronidase	SuC, ERT
Multiple sulfatase deficiency	Sulfatase modifier protein	SuC
Niemann-Pick Type A	Sphingomyelinase	SuC, HSCT
Niemann-Pick Type B	Sphingomyelinase	SuC
Niemann-Pick Type C1	NPC1	SuC, HSCT
Niemann-Pick Type C2	NPC2	SuC
Pompe	Acid alpha-glucosidase	SuC, ERT
Pyknodysostosis	Cathepsin K	SuC
Sialic acid storage disease, Salla	Transport protein	SuC

pair the mutated gene, a constant expression of the concomitant protein can be achieved, leaving a life-long expression of the gene that can potentially restore the phenotype. The challenges of gene therapy lie within the wide range of target tissues. Persistent expression of the target gene has to be achieved in widely diverse tissues such as the central nervous system (CNS), skeletal and cardiac muscle, liver, kidney, eye, and bone. Diseases with CNS involvement require a different approach than LSDs without CNS involvement. The pros and cons of different gene therapy strategies will be discussed in more detail.

This thesis is focused on the development of *ex vivo* lentiviral gene therapy in Pompe's disease using hematopoietic stem cells (HSCs) as a transfer vehicle.

LYSOSOMAL FUNCTION

Lysosomes are considered the digestive system of the cell and contain around 50 different hydrolases. The lysosomal hydrolases function optimally in the acidic environment of the lysosomal interior (pH 4.0 – 5.0). They include proteases, nucleases, lipases, sulfatases, glycosidases, phospholipases and phosphatases and have a variety of digestive functions. For example degradation of extra- and intracellular molecular compounds, destruction of phagocytosed microorganisms, and by doing so these hydrolases also provide nutrients to the cell⁵. Most hydrolases are targeted to the lysosomes by exposure of mannose 6-phosphate residues on carbohydrate side-chains that are in turn recognized by mannose 6-phosphate receptors in the trans Golgi network (Figure 1).

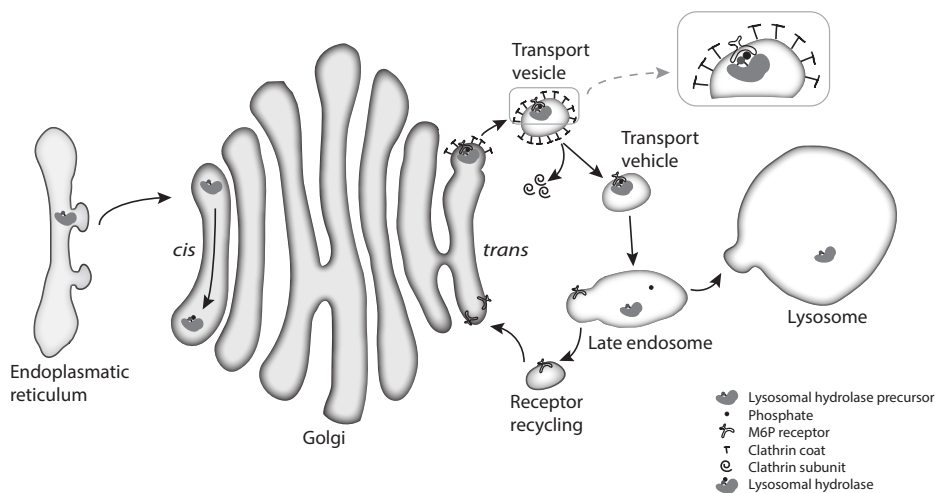


Figure 1: Intracellular hydrolase maturation (not to scale, figure based on⁸).

The mannose 6-phosphate group is added to N-linked oligosaccharides by two enzymes that act sequentially to catalyze the addition of the mannose 6-phosphate (M6P) group; GlcNAc phosphotransferase and N-acetylglucosamine-1-phosphodiester α -N-acetylglucosaminidase. GlcNAc phosphotransferase recognizes the lysosomal hydrolases and adds GlcNAc-phosphate to one or two of the mannose residues on each oligosaccharide chain. The N-acetylglucosamine-1-phosphodiester α -N-acetylglucosaminidase cleaves off the covering GlcNAc residue to produce a mannose 6-phosphate group. In general, most of the hydrolases have more than one oligosaccharide side-chain, which allows more than one mannose 6-phosphate residue to be added, increasing the affinity for the mannose 6-phosphate receptor⁸.

After binding to the mannose 6-phosphate receptor the hydrolases are encapsulated in clathrin-coated vesicles that bud off from the trans Golgi network and are transported to the late endosomes. There, receptor and ligand uncouple due to lowering of the pH. The ligand travels on to the lysosomes by vesicular transport and the mannose 6-phosphate receptor is in a similar way transported back to the Golgi system guided by a signal peptide in the cytoplasmic tail of the mannose 6-phosphate receptor⁸.

Substrates enter the lysosomes by autophagy. Autophagy is a membrane trafficking process responsible for the delivery of cytoplasmic components to the lysosomes. Vesicles with a double-membrane, called autophagosomes, mediate this pathway. The lysosomes fuse with the autophagosomes, they release their hydrolytic enzymes into the resulting autolysosome, and the content of the autophagosome is degraded. Currently, autophagy attracts a lot of attention for its role in the pathophysiology of LSDs⁸⁻¹⁰.

Mutations in genes coding for one of the lysosomal hydrolases or for other proteins involved in lysosomal structure or function result in lysosomal storage disorders. The common principle is: undegradable molecular substances accumulate inside the lysosomes.

A very severe example of a lysosomal storage disorder is inclusion-cell disease (I-cell disease) named after the numerous inclusions that are present in skin cells (fibroblasts). I-cell disease is a very rare disorder in which almost all of the lysosomal enzymes are missing due to the deficiency of the phosphotransferase that normally adds mannose 6-phosphate to the lysosomal hydrolases. Thus, instead of being transported to the lysosomes, the hydrolases are secreted. Clinical symptoms of these patients share similarities with MPSI, and patients with the most severe I-cell phenotype usually succumb before the age of seven years due to congestive heart failure or recurrent respiratory tract infections^{2,11}.

POMPE'S DISEASE

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Pompe's disease is one of the 12 presently known glycogen storage disorders (GSDs) and is a lysosomal glycogen storage disorder (Table 1 and 2). The GSDs are inherited metabolic disorders of glycogen metabolism. Glycogen is composed of more than 60,000 glucose moieties. The glucose molecules are arranged in straight chains by α -1,4 linkages and branching points by α -1,6 linkages. Glycogen is an important storage of energy in all tissues, most profoundly in liver and muscle. Many enzymes are involved in the degradation and synthesis of glycogen². Aberrations in these enzymes can lead to deficiencies in glycogen metabolism, which causes glycogen storage disorders (Figure 2).

GSDs primarily affect the liver and/or muscle tissue. The different types are enlisted in Table 2. Pompe's disease (GSD II) is the only disease for which a causal therapy, ERT is available. GSD 0, I and III can be kept under control with a specific diet.

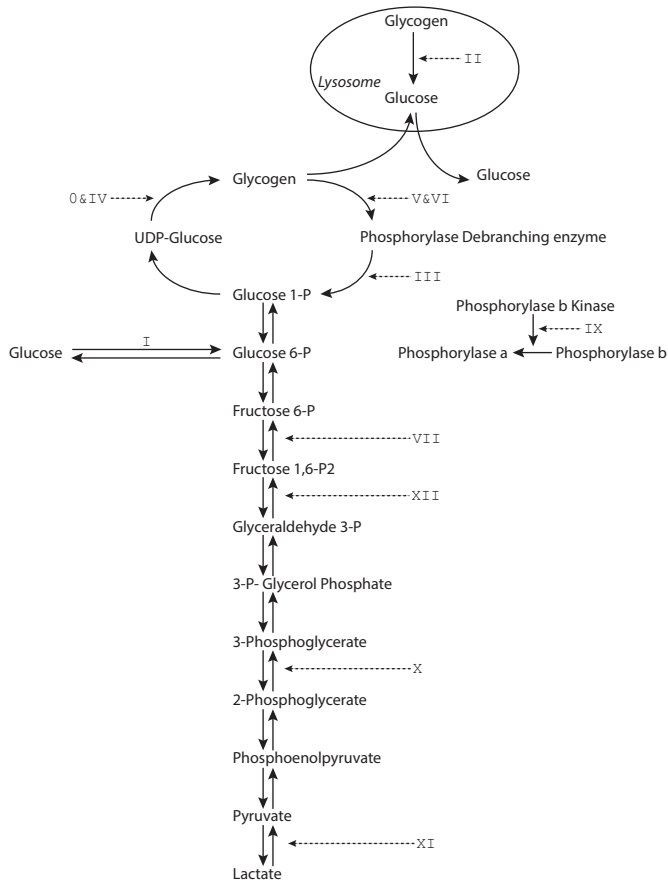


Figure 2: Complex pathway of glycogen synthesis and degradation with the different glycogen storage disorders indicated (0-XII, table 2, based on²).

Table 2: Overview of glycogen storage disorders, the defective enzyme and treatments options. SuC, supportive care; ERT, enzyme replacement therapy².

Glycogen storage disorder	Defective enzyme	Treatment options
Type 0, Lewis	Glycogen synthase	SuC
Type I, von Gierke	Glucose-6-phosphatase	SuC,
Type II, Pompe	Acid α -glucosidase	SuC, ERT
Type IIa, Danon	LAMP2	SuC
Type III, Cori	Amylo-1,6-glucosidase	SuC
Type IV, Anderson	Amylo-1,4-1,6-transglucosidase	SuC
Type V, McArdle	Myophosphorylase	SuC
Type VI, Hers	Phosphorylase (liver)	SuC
Type VII, Tarui	Phosphofructokinase	SuC
Type IX	Phosphorylase kinase	SuC
Type X	Cyclic 3',5' AMP-dependent kinase	SuC
Type XI, Fanconi-Bickel	Glucose transporter 2	SuC
Type XII	Aldolase A	SuC

Pompe's disease (Glycogen storage disease type II, GSD II, acid alpha-glucosidase deficiency, acid maltase deficiency, OMIM # 232300) is the only GSD in which individuals are deficient in a lysosomal hydrolase¹². The hydrolase acid alpha-glucosidase (GAA) is deficient, which leads to abnormal accumulation of glycogen in the lysosomes of virtually all cell types in the body, most prominently in the myocytes of skeletal, smooth and cardiac muscle (Figure 3). The function of GAA is to catalyze the hydrolysis of all alpha-1,4 and alpha-1,6 glycosidic linkages of glycogen. Insufficient degradation of glycogen in the lysosomes causes the organelle to swell and to burst eventually. The following release of lysosomal hydrolases into the cytoplasm is thought to contribute to the pathologic process. It results in cell and tissue damage and eventually organ failure.

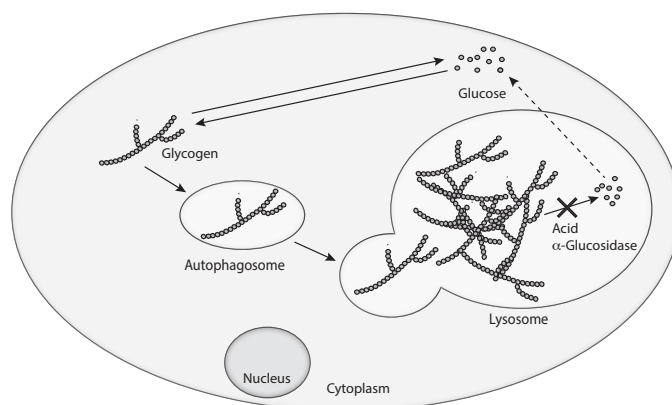


Figure 3: Intra-lysosomal glycogen accumulation (not to scale).

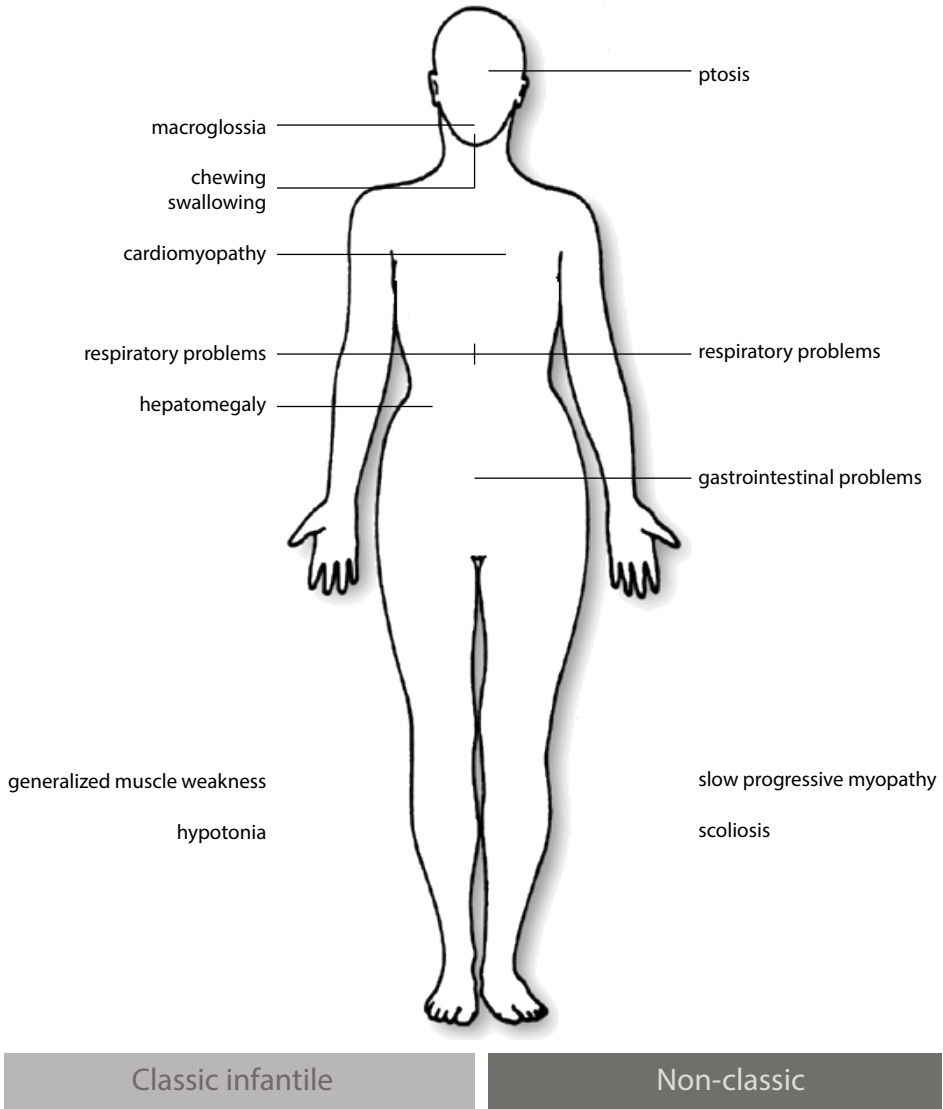


Figure 4: Symptoms in classic and non-classic types of Pompe’s disease.

CLINICAL FEATURES OF POMPE’S DISEASE

Though there is a wide variety of disease severity and progression, all Pompe’s disease patients display glycogen accumulation in almost every tissue but storage in muscle is most prominent. In the long term it leads to organ dysfunction, morbidity and shortened life expectance. The disease severity is primarily determined by the severity of the pathogenic mutations in the acid alpha-glucosidase gene, although modifying factors can play an important role, and further depends on disease duration^{12,13}.

The Dutch pathologist Dr. J.C. Pompe first described Pompe's disease in 1932. He reported a 7 months old girl who succumbed to severe cardiac hypertrophy¹⁴. This case typically represents the classic-infantile form of Pompe's disease, the most severe form of Pompe's disease. Symptoms in these patients typically display immediately after birth, with a median onset of 1.6-2.0 months and typically relate to the involvement of cardiac and skeletal muscles and include cardiorespiratory failure, hypotonia, feeding difficulties and failure to thrive. These patients usually have a GAA activity of less than 1%. As Dr. J.C. Pompe described, the most characteristic feature discriminating between classic-infantile and other forms of Pompe's disease is the profound cardiac involvement. Patients with classic-infantile Pompe's disease show a very thick left ventricular wall and septum, which results in a weak function of the heart and can lead to death if the pump function fails^{15,16}.

Most babies with Pompe's disease have a significant head lag and are unable to reach major motor milestones such as sitting and rolling over due to their muscle weakness¹². Other clinical features in the infantile form can include macroglossia and hepatomegaly, while the respiratory muscles are always involved (Figure 4)¹⁶. Untreated patients with classic-infantile Pompe's disease rarely survive beyond 1 year with a mean age of death between 6.0-8.7 months¹⁷. Due to the life saving effect of enzyme replacement therapy, other clinical features can be added to the list of symptoms seen in classic-infantile Pompe's disease, such as osteoporosis and osteopenia^{18,19}. Hearing difficulties are caused by morphological changes due to the glycogen storage present in the middle ear, inner ear and auditory nervous system^{20,21}.

Glycogen storage in the central nervous system (CNS) has been observed in patients with classic-infantile Pompe's disease, however, this storage does not seem to impair the cognitive function of patients with Pompe's disease²². The long-term effects need to be awaited now that patients with classic-infantile Pompe's disease have an extended life expectancy thanks to the introduction of ERT.

Other forms of Pompe's disease (non-classic) are characterized by a less progressive disease course without cardiac involvement and storage in the brain (Figure 4)^{15,16}. The symptoms can present at any age. The patients have residual acid alpha-glucosidase activity in a range from 3 to maximally 30% of average normal activity levels in fibroblasts. In these patients, the disease is characterized by progressive muscle weakness. It mainly affects the limb-girdle muscles. Respiratory insufficiency results from weakness of the diaphragm and intercostal muscles. Patients with non-classic Pompe's disease eventually become wheelchair and ventilator dependent. The most frequent cause of death is respiratory failure²³⁻²⁶.

Diagnosis

Laboratory methods to diagnose Pompe's disease usually combine biochemical assays and DNA analysis²⁷⁻²⁹. The quickest procedure is the use of leukocytes isolated from peripheral blood. Deficiency of alpha-glucosidase activity can easily be established by using glycogen as substrate or an artificial substrate provided that acarbose is added to the reaction mixture to inhibit the activity of interfering glucosidases³⁰⁻³². An assay in dried blood spots is also possible but less reliable and better suited for screening purposes³³⁻³⁵. The most sensitive method to diagnose Pompe's disease is the measurement of GAA activity in fibroblasts obtained by skin biopsy^{22,32}. The skin cells are brought into a cell culture system and expanded, which can take up to 4-6 weeks and causes diagnostic delay. The enzyme assay can also be performed in a muscle biopsy homogenate, but muscle biopsies are in practice far more useful for studying muscle pathology and lysosomal glycogen storage using the PAS staining procedure²². Of note, the location of the muscle biopsy can greatly influence the outcome; not all muscle fibers at all locations are equally affected. This problem holds for all Pompe phenotypes except classic-infantile Pompe's disease.

Mutation analysis is of great importance for understanding the genotype-phenotype correlation in Pompe's disease. It is the only way to reliably detect Pompe's disease carriers and to counsel family members of patients properly.

Pompe's disease is a very rare disorder and many of the symptoms are shared with other medical conditions that should be included in the diagnosis²². Careful evaluation of the symptoms in combination with enzymatic testing should exclude any other diseases.

Genetic heterogeneity

Pompe's disease is an autosomal recessive disorder; both *GAA* alleles need to carry a pathogenic sequence variation for the disease to manifest. The *GAA* gene (OMIM # 606800) is located on chromosome 17q25.2-q25.3 and encodes a cDNA of 3.6kb. At present, 453 sequence variations are known⁴⁰. They are randomly spread over the 20 exons and introns of the *GAA* gene. Most mutations are unique and very rare. However, some occur more frequently such as c.-32-13T>G which is found in 50% of the children and 80-90% of the affected adults in the Caucasian population. c.-32-13T>G is never found in infants with classic-infantile Pompe's disease. It has been shown that patients with the same c.-32-13T>G haplotype can have a quite variable age of onset and rate of disease progression. It is therefore concluded that genetic and environmental modifying factors have a great impact on the course of Pompe's disease⁴¹.

Patients with a classic-infantile disease presentation have on each of their two *GAA* alleles a mutation that leads to complete loss of *GAA* function, either by interference with protein

synthesis, posttranslational modifications and/or function. Patients with other forms of Pompe's disease have almost always one allele with a less severe mutation that allows some level of residual GAA expression and activity, which explains their milder phenotype.

Treatment options

Until 10 years ago, the management of Pompe's disease was solely based on supportive care. After the development of enzyme replacement therapy for Pompe's disease and after the approval of this approach by the European Medicines Agency (EMA) and U.S. Food and Drug Administration (FDA) in 2006, treatment options for Pompe's disease patients significantly improved. This was the beginning of a new era for Pompe's disease patients.

ENZYME REPLACEMENT THERAPY

Background

Enzyme replacement therapy is based on the principle that intravenously administered lysosomal enzymes can reach the affected tissues and are taken up by the cells via endocytosis whereby they gain access to the lysosomes where they are needed⁴²⁻⁴⁵. The mannose 6-phosphate receptor pathway is instrumental in this process, like it is for the intracellular transport of most lysosomal proteins. The process whereby a small portion of the newly synthesized lysosomal enzymes is secreted during transit to the lysosomes and subsequently internalized by adjacent cells is called cross-correction. This mechanism is also considered of great importance for hematopoietic stem cell (HSC) mediated gene therapy. The idea behind this approach is that only a certain cell type needs to be targeted while distant tissues can be cured by cross-correction and will be discussed later.

The recombinant enzyme that is currently used for ERT in Pompe's disease was first produced in rabbit milk and later in Chinese Hamster Ovarian (CHO) cells⁴⁶⁻⁴⁸. The efficacy of ERT in classic-infantile Pompe's disease is remarkable in that the cardiac hypertrophy and ventricular function respond generally well⁴⁹. Most patients are still alive after 1 year of treatment, while untreated patients have a median survival of 6-8 months. Some of the treated infants are able to sit and walk. It is widely believed that very early start of treatment leads to the best results.

A recent study has shown that the cognitive development of children with the classic-infantile form of Pompe's disease who received ERT, is normal to mildly delayed⁵⁰. The authors noted that cognition is easily underestimated in affected children under 5 years because of poor motor function.

The results of ERT are less striking in adults with Pompe's disease due to the naturally slow disease progression. However, it has been shown that ERT has a significant effect on the distance walked, pulmonary function and survival. It does not guarantee symptom-free survival; long-term follow-up results are still pending^{46,51-54}.

Immune response

With regard to immune responses, patients with Pompe's disease can be categorized by the virtue of being 'cross-reactive immunologic material (CRIM) positive or negative'^{38,39}. The CRIM status refers to the patient's ability to produce GAA. Patients who do not produce any form of GAA, due to their very severe mutations in both GAA alleles, are designated CRIM-negative as opposed to CRIM-positive patients that do produce some GAA protein. Most patients who are CRIM-negative develop immune reactions against the 'foreign' recombinant enzyme. In some particular cases the antibody titer may reach such a high level that it neutralizes the activity of the therapeutically administered recombinant human enzyme or its uptake by the affected cells^{36,38}. Also CRIM-positive patients can develop anti-GAA antibodies³⁶⁻³⁹.

The combination of adverse effects and immune response may create a situation in which ERT is no longer effective. This situation has been encountered in a minority of both CRIM-negative as well as CRIM-positive patients. To overcome this problem, different strategies are being studied. For example, the use of immune modulating agents, like methotrexate and Rituximab are being studied to determine whether they can suppress the immune system and enable the continuation of ERT⁵⁵. Another strategy is to induce immune tolerance to the foreign protein before the administration of ERT. Hematopoietic stem cell transplantation (HSCT) is known to induce immune tolerance due to its intrinsic nature⁵⁶. An autologous HSCT can be used in combination with gene therapy, which will be discussed later in this thesis, or an allogeneic HSCT can be used. This strategy has proven its value in MPS I, an LSD with neurological involvement⁵⁷⁻⁵⁹. The few attempts at allogeneic HSCT in Pompe's disease have been disappointing. The enzyme levels provided by the donor hematopoietic stem cells (HSCs) were simply too low to be of any therapeutic effect and the patients had already a profound storage of glycogen at the start of the treatment^{60,61}.

ONGOING RESEARCH

Chaperones

To become fully active, enzymes must be folded in a unique way. ER localized chaperones assist the cells in protein folding and translocation from the endoplasmic reticulum (ER) to their destination, e.g. the lysosomes^{62,63}. Small molecular chaperones can also stabilize

therapeutic proteins and make ERT more efficient. In Fabry's disease and in Pompe's disease this is seen as a challenging new way to increase the efficacy of ERT. A Phase II open-label, multicenter clinical trial sponsored by Amicus Therapeutics is currently ongoing with the oral intake of molecular chaperone (AT2220) at the same time that the patients receive ERT for Pompe's disease^{64,65}. The initial results show an increase in plasma GAA in all patients compared to plasma levels in the absence of AT2220⁶⁶.

Substrate reduction therapy

Substrate reduction therapy (SRT) has shown to be effective in other lysosomal storage disorders, like Fabry disease and Niemann-Pick disease^{67,68}. This approach is aimed to reduce the production and lysosomal influx of the enzyme's substrates⁶⁹. Recently, Richard and colleagues applied this strategy in a model system employing myoblasts obtained from a *Gaa* deficient mouse model⁷¹. Using antisense mRNA technology they inhibited the expression of glycogenin (GYG) initiating glycogen granule formation and glycogen synthase (GYS) catalyzing the 'growth' of glycogen. A strong reduction of lysosomal glycogen accumulation was found.

Besides the strategies described above, many others are being pursued, for example to increase the mannose 6-phosphate modification of GAA to obtain a more efficient uptake of the enzyme⁷⁰, but a full discussion lies beyond the scope of this thesis.

The next session of the Introduction will involve gene therapy and its application in Pompe's disease.

GENE THERAPY

Background

Early in the twentieth century, thoughts of correcting inherited traits by gene modification were emerging. Breakthroughs in genetics, biochemistry and molecular biology have changed the perspective of gene therapy so that it has become a near certainty that inherited diseases can be corrected by gene modification in the future.

There are two ways to deliver genetic material to the host; *in vivo* and *ex vivo*. In *in vivo* gene therapy, the DNA is directly transferred to the patient by the use of microparticles (like liposomes) or genetically modified viruses. The major drawback of this approach is the fact that there is no control over the transgene and the target tissue might not be easily reached and antibody formation against the foreign protein can occur. The very first documented *in vivo* gene therapy trial (1975) was performed in patients with hyperargininemia, in which the patients were intravenously injected with a Shope papillomavirus

that produces arginase, an enzyme that decreases the arginine concentration in serum of rabbits and other animals. However, when applied to patients, no reduction of arginine was observed⁷². Although this was considered as a failed gene therapy attempt, it did boost the scientific community to believe gene therapy could become a reality.

Ex vivo gene therapy is the alteration of the DNA of a specific type of cell outside the patient's body, usually with a viral vector. This strategy requires often a surgical removal of the patient's own target cells, e.g. bone marrow or muscle cells, in order to obtain a sufficient cell population to modify the cells. The greatest advantage of *ex vivo* gene therapy over *in vivo* gene therapy is the substantial reduction of graft-versus-host rejections, as often seen in allogeneic bone marrow transplantations. This minimizes the transplant related morbidity and mortality rate. Other advantages are the possibility to expand the target cell population *ex vivo*, which reduces the size of the biopsy needed to obtain a sufficient amount of cells and the ability to control transgene expression. Having said this, small alterations of the target cells can occur when they are placed outside the body in a culture dish and the expansion can be extremely difficult for some cell types as HSCs and muscle stem cells (MuSCs)⁷³⁻⁷⁵.

HSCs as transfer vehicle

As mentioned above, hematopoietic stem cells (HSCs) are an attractive target cell population for the treatment of inherited metabolic diseases. The unique features of the HSCs include the ability to self-renewal and differentiation in every cell type of the hematopoietic lineage. HSCs can be obtained from bone marrow (BM), mobilized peripheral blood or umbilical cord blood (UCB). HSCs can be mobilized to the peripheral blood by the use of cytokines or adhesion molecules; these extract the HSCs from their microenvironment in the bone marrow and send them to the peripheral blood, from which they can be isolated. Cytokines such as granulocyte colony-stimulating factor (G-CSF) activate neutrophils and osteoclasts resulting in the release of stem-cell-factor (SCF), proliferation of progenitor cells and activation or degradation of adhesion molecules^{76,77}. Other chemokines such as interleukin-8 and stromal cell-derived factor-1 also play a major role in mobilization of progenitors^{78,79}. The most commonly used HSC mobilizing cytokine is G-CSF, in most cases this agent is administered for a few days, after which the HSCs are isolated from the blood⁸⁰. Umbilical cord blood cells are currently considered to be the best source of HSCs. They have the highest percentage of HSCs that are capable of reconstituting the hematopoietic niche; three times higher than matched bone marrow and even six times higher than HSCs obtained from mobilized peripheral blood⁸¹. However, using any of the three available methods the isolation of sufficient numbers of HSC remains a challenge. Ways to expand HSCs *in vitro* are vividly explored, but it remains extremely difficult to obtain robust expansion while maintaining the quiescence of the HSCs. A major advantage of using HSC methodology in gene therapeutic approaches is that HSCs are able to

cross the blood-brain barrier. Myelomonocytic cells in particular migrate to the brain and differentiate into microglial cells. This is very important in LSDs with CNS involvement, since intravenously administered enzyme cannot cross the blood-brain barrier. In LSDs like Mucopolysaccharidosis type I, HSCT is the preferred choice of treatment to prevent or stop the neurodegenerative process, which leads to early death in childhood. However, the effect of HSCT alone is not sufficient to halt the disease process. Therefore, HSCT is currently applied in combination with ERT.

In theory it should be possible to life-long correct genetic diseases because of the self-renewal abilities of HSCs and the expression of the transgene in all the hematopoietic lineages. This can lead to correction of a broad variety of target cells in the body. Due to the enormous proliferative potential of HSCs, an integrating vector is required to maintain life-long gene expression and disease correction.

Hematopoietic stem cells were the first cells to pursue *ex vivo* gene therapy with. HSCs are particularly suitable for gene therapy, because of their involvement in many hematopoietic disorders; they are relatively easy to retrieve from patients and can be manipulated *ex vivo* (Figure 5). Their outstanding capability to engraft into the bone marrow after reintroduction to the patient is necessary for successful treatment of many disorders.

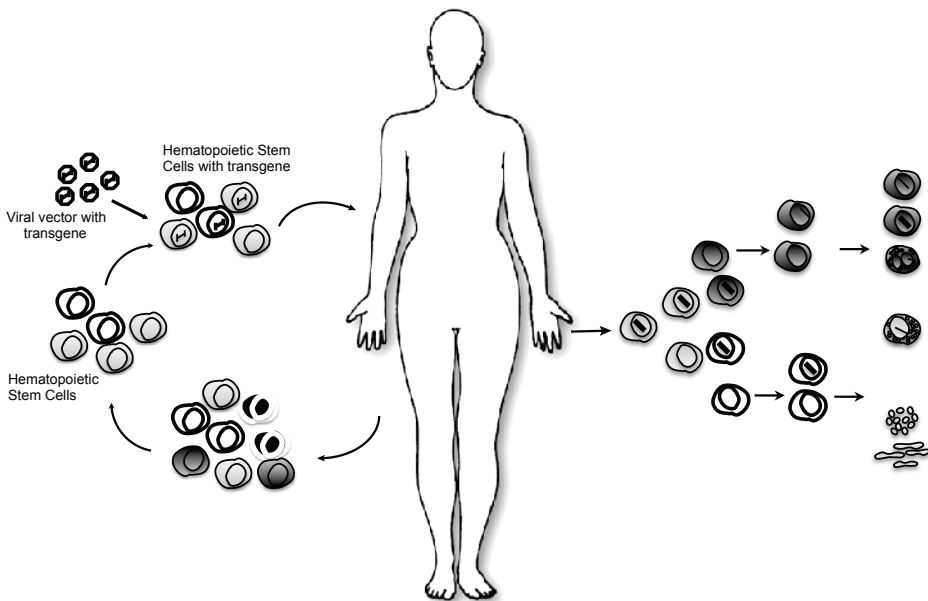


Figure 5: HSCs transplantation strategy.

The first successful gene therapy trial with genetically modified HSCs involved a gamma-retrovirus to insert a neomycin resistance gene *ex vivo* in HSCs of cancer patients. After infusion of the modified HSCs, expression of neomycin resistance could be demonstrated in hematopoietic progenitors⁸². The major breakthrough in gene therapy with genetically modified HSCs was the successful correction of disease in patients with X-linked severe combined immunodeficiency (X-SCID)⁸³. X-SCID is a rare disease, affecting the immune system in which there is no T-cell development due to a mutation in the gene expressing interleukin-2 receptor common γ -chain (IL2RG). In this particular trial, the patients' hematopoietic stem cells were genetically modified with a retroviral vector, expressing the IL2RG gene. It is considered as a success in the field, since all patients maintained their acquired immune functions for more than 9 years. At present all patients, except 1, live under normal, non-protected, conditions⁸⁴⁻⁸⁶. On the other hand, the trial was a warning against the use of integrating vectors, since 5 out of 20 patients in this trial developed leukemia as a consequence of retroviral vector integration at an unfavorable site, which led to activation of proto-oncogenes^{87,88}. These findings have led to a more cautious attitude towards the use of integrating vectors and have initiated many studies regarding the sites of integration of retroviral vectors, the identification of potential integration sites and methods to influence the sites of integrations of the different vectors.

Viral vectors

Viruses have been modified to serve as a vehicle for gene transfer. Depending on the target cell different types of viruses are being used. Given the enormous diversity of disease entities and their associated target tissues, it is obvious that there will not be a single

Table 3: Viral vectors used for gene therapy. The differences in capacity, integration ability, expression of the transgene and the advantages and disadvantages of the viral vectors are shown here.

Virus	Capacity	Integration	Expression	Advantages	Disadvantages
Adeno-associated virus	+/- 4.5-9 kb	low +/-0.1-1%	Long term	Episomal, infects nondividing cells	Immunogenic, toxicity
Adeno virus	2-38 kb	No	Short term	Efficient gene transfer	Immunogenic, transient
Alphavirus	+/- 5 kb	No	Short term	Broad host range, high expression	Virulence
Herpes simplex virus	+/- 30 kb	No	Short term, except in CNS	Neurotropic, large capacity	Virulence, persistence in neurons
Lentivirus	7-18 kb	Yes	Long term	Stable integration, infects nondividing and terminally differentiated cells	Insertional mutagenesis
Retrovirus	1-7.5 kb	Yes	Long term	Stable integration	Insertional mutagenesis, requires cell division
Vaccinia virus	min 25 kb	No	Short term	Broad host range, high expression, large capacity	Immunogenic, transient

vector suitable for all diseases. At present, the viral vectors suitable for clinical applications are: adenovirus (mostly used in cancer related gene therapy), adeno-associated virus, gamma retrovirus and lentivirus (Table 3). Some viral vectors, like the ones derived from retroviruses, insert a part of their genome into the host cell whereas others, such as the adeno-associated viruses (AAVs), are considered to be non- to low-integrating viruses. Each viral vector has its advantages and disadvantages somewhat dependent on the type of disease.

Currently, the most frequently used viral vectors for gene transfer are adeno-associated virus vectors. They belong to the Parvoviridae and are small, non- enveloped viruses that harbor single stranded DNA. In general AAV needs another virus, e.g. adenovirus, for productive infection of the host cell. Up to date, 12 different human serotypes have been identified (AAV-1 to AAV-12) and together with a lack of pathogenicity this increases the potential of the use of AAV for genetic transfer. In the quest to find the ultimate vector and the best target cells for the treatment of Pompe's disease different approaches are currently under investigation. Most of the gene therapy research conducted in Pompe's disease has been performed with AAV vectors. Researchers deliver the therapeutic GAA cDNA incorporated in an AAV vector to a specific peripheral tissue, like diaphragm or lower limb. This does not only lead to a limited local delivery of the enzyme, it also has other drawbacks like low transduction efficacy of the tissue, a high risk of immune responses (to the alien transgene or the vector capsid or due to pre-existing neutralizing antibodies the patient has acquired during life) and finally, cell division dilutes the integrated therapeutic gene in the genome of the cells, thereby losing the efficacy of the therapy.

To effectively study any gene modifying treatment *in vivo*, animal models are extremely important. In the case of Pompe's disease, the mouse *Gaa* gene has an 84% homology with the human gene. Several mouse models with *Gaa*-deficiency have been generated^{89,90}. In 1998, the first mouse model with a targeted disruption of the *Gaa* gene was published⁸⁹. Consequently, no *Gaa* mRNA is produced and *Gaa* levels are not detectable in tissues of the *Gaa*^{-/-} mice. The absence of the *Gaa* leads to a progressive generalized glycogen storage. These mice present the generalized glycogen storage as well as functional impairment of skeletal muscle. They also display a profound cardiomyopathy and altered cardiac and respiratory parameters like patients with classic-infantile Pompe's disease⁹¹. Other mouse models described show either similar phenotypes as mentioned above or display a milder phenotype⁹⁰.

In a *Gaa*^{-/-} mouse model, both systemic delivery and delivery to an isolated tissue of an AAV vector with the therapeutic GAA gene have mediated partial biochemical correction of the phenotype. However, the success of this treatment highly depends on the absence of a humoral immune response against the therapeutic GAA. To obtain immune tolerance to

the foreign protein or vector capsid, immune-suppressive drugs need to be administered. Another way to achieve immune tolerance in AAV treatment is to use a liver-specific AAV (AAV2/8 or AAV2/9), since hepatocyte transgene expression can lead to transgene specific immune tolerance by the TGF- β -dependent induction of Treg cells, that suppress T and B cells^{92,93}.

The strategy we propose for Pompe's disease involves the efficient transduction of HSCs with integrating lentiviral vectors. Since the HSCs and their progeny undergo many cell divisions, a stable integrating vector is therefore required, which leads to a continuous supply of the enzyme to the whole body and results in mice demonstrating a lack of immune response to the recombinant GAA protein⁹¹.

Retroviral vectors

Retroviruses and lentiviruses both belong to the *Retroviridae* family.

Retroviral vectors have been used for decades in the field of gene therapy. With their ability to insert their genetic content in the genome of the host and to use the hosts' transcription and translation machinery to replicate their own genome in a wide variety of cells, like HSCs and neural stem cells, they have become attractive candidates for the treatment of inherited monogenetic disorders. Other advantages of retroviral vectors are the relative easiness to obtain sufficiently high viral titers for efficient gene transfer and their capacity to accommodate rather lengthy expression cassettes. All of these aspects are prerequisites for efficient and long-term gene expression and essential elements to achieve therapeutic efficacy. Besides these advantages, some disadvantages play a role in the use of retroviral vectors. The major limitation of the retroviral vectors is that they cannot transduce non-dividing cells; they require cell division whereby the nuclear membrane resolves and the way to integration in the host genome opens. Other disadvantages are the instability of some retroviral vectors and the risk of insertional mutagenesis by viral integration.

Retroviruses possess two identical single-stranded RNA copies that are reverse transcribed into a linear double-stranded DNA copy, which subsequently integrates in the host genome. The retroviral genomes consist of the genes encoding the transcriptional units *env*, *gag*, *pol* and *pro*. The gene *env* is responsible for the surface glycoprotein and for other transmembrane proteins enabling binding to cellular membrane receptors. *Gag* encodes the structural protein Gag involved in the assembly of non-infectious and immature viral-like particles. *Pro* encodes the viral protease that facilitates maturation of viral particles, while *pol* encodes reverse transcriptase and integrase.

To assemble a retroviral vector, it is necessary to separate the promoter and gene of interest (transfer vector) from the structural genes required for virus generation. In this way, the

viral vector contains besides the promoter and gene of interest all the sequences necessary for translation and transcription, but cannot replicate.

After the previously discussed X-linked SCID retroviral gene therapy trial resulted in a T-cell acute lymphoblastic leukemia (T-ALL) in a number of patients, which was related to the vector insertion site, the development of safe gene transfer vectors became even more urgent.

Other integrating viral vectors were evaluated. HIV-based lentiviral vectors were considered more appealing for use in gene therapy, since they do not display any preference towards transcription start sites and harbor additional safety features^{94,95}.

Lentiviral vectors

The lentiviral vectors most commonly used for preclinical research are based on the human immunodeficiency virus-type 1 (HIV-1), a member of the retrovirus family. The wild-type HIV-1 genome is considered complex since it also contains some accessory genes; *nef*, *tat*, *rev*, *vif*, *vpr* and *vpu*. These accessory genes are known to play a role in replication and pathogenicity⁹⁶⁻⁹⁸. The lentiviral vectors have undergone quite some modifications over time. The first generation lentiviral vectors consisted of 3 packaging plasmids, expressing all viral ORFs including the Env-coding plasmid driven by the CMV promoter. Two wild type long terminal repeats (LTRs, regulatory regions for transcription initiation and polyadenylation) framed the expression cassette, and the vector was further composed of Rev and Rev-Responsive Element (Rev acts by binding to RRE to promote nuclear export, stabilization and utilization of the viral mRNA) and sequences responsible for genome packaging and reverse transcriptase^{99,100}.

The first generation lentiviral vectors used a three-plasmid system. One plasmid contained the packaging signal, internal promoter and two wild type LTRs. A second plasmid provided the accessory genes *gag*, *nef*, *pol*, *rev*, *tat*, *vif* and *vpr* and a third plasmid contained the envelope gene. High viral titers were obtained¹⁰¹.

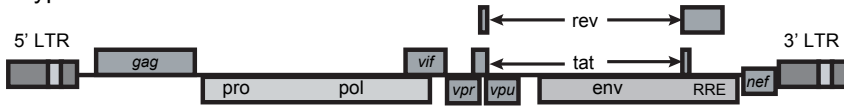
For the second generation lentiviral vectors, most of the accessory genes were removed (*nef*, *vif*, *vpr* and *vpu*, all promote HIV virulence), only *tat* and Rev remained within the vector. The removal of the accessory genes did not cause any drop in viral titers; they remained high¹⁰².

In third generation vectors, *tat* and Rev were removed, and Rev was provided by a fourth plasmid. (Figure 6)

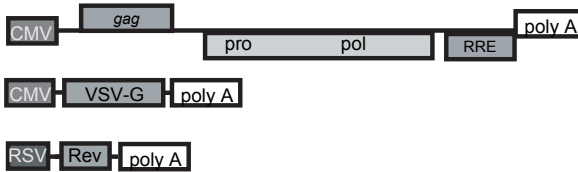
The removal of *tat* did not influence the viral titers when the U3 region of the 5' LTR promoter was replaced by a promoter from another virus like cytomegalovirus (CMV)¹⁰³.

Other safety features build in the HIV-based lentiviral vectors are aimed to avoid replication and increase the gene transfer efficacy.

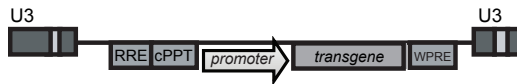
Wild type HIV-1



Packaging plasmids



Transfer plasmid



Transfer vector



Figure 6: Scheme of HIV-1 based lentiviral vector.

The development of self-inactivating (SIN) lentiviral vectors renders the capacity of the vector to transcribe mRNA from the LTR. Because of a deletion in the 3' LTR promoter region, the LTR promoter in the 5' position is removed by reverse transcriptase so that full-length transcription of the vector is prevented when the vector integrates in the host genome. Without transcription from the LTR the viral genome can be packaged into a viral particle¹⁰³⁻¹⁰⁶.

The envelope protein used in most lentiviral vectors is based on VSV-G (Vesicular Stomatitis Virus-G). Because its envelope protein binds to a receptor in the phospholipid bilayer of the cytoplasmic membrane of vertebrates and invertebrates it provides a very broad tropism¹⁰⁷.

Other features include the addition of the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), to improve viral titers and expression of the transgene. Furthermore the vector contains a central polypurine tract (PPT) to enhance reverse transcription; these vectors constitute the third generation lentiviral vectors and are unable to form wild-type HIV^{108,109}.

One of the drawbacks of a lentiviral vector system is the highly complicated production system, which makes it difficult to produce the virus in such a way that the titers are sufficiently high for efficient transduction of the target cells. To achieve a high transgene expression, the use of more viral particles is necessary, or the level of expression of the transgene needs to be optimized. The use of more viral particles is not desirable due to the higher chance of insertional mutagenesis. Besides the optimization of the vector backbone as described above, the DNA sequence can be codon-optimized. This frequently applied technique is used to enhance the expression of a certain protein by increasing the transcription efficacy of its gene or cDNA. It also results in increased mRNA stability, mRNA export and improved translation efficacy¹¹⁰⁻¹¹². To accomplish this, codon sequences with a low frequency are replaced by codon sequences with high frequencies. The amino acid sequence stays the same, only the codon sequence is altered, and gene expression can be increased significantly in most cases¹¹³⁻¹¹⁵. Besides enhancing gene expression, codon optimization removes cryptic splice sites and eliminates secondary RNA structures and instability motifs.

Safety concerns

The main reason that lentiviral vectors have attracted interest for therapeutic purposes is their integration into a host genome effectuates a long lasting cure. Paradoxically, this favorable feature can be intrinsically harmful, which was exemplified in the SCID-clinical trial with retroviral mediated gene therapy. Vector-mediated insertional mutagenesis has also been observed in clinical trials regarding Chronic granulomatous disease and Wiskott-Aldrich syndrome, but all of these trials were conducted with first generation γ -retroviral vectors^{87,88,116,117}. The latest clinical trials with integrating viral vectors involves a third generation lentiviral vector for the treatment of X-linked adrenoleukodystrophy (ALD), metachromatic leukodystrophy (MLD) and Wiskott-Aldrich syndrome (WAS) and revealed a more polyclonal integration pattern^{118,119, 125, 126}.

In general, the integration pattern of lentiviral vectors is largely stochastic, with no good prediction where in the genome the integration will take place¹²⁰.

Current status gene therapy clinical trials

At the time of writing, the European Medicines Agency (EMA) or U.S. Food and Drug Administration (FDA) have not approved any lentiviral gene therapy product for clinical application. Around 60% of the ongoing clinical trials involving gene transfer are still in Phase I, whereas 20% are in Phase II, a small proportion (0.2%) is approaching Phase IV. Most of the trials use adenovirus, and only 2.9% entail lentiviral vectors.

The first gene therapy clinical trials with retroviral vectors for LSDs were completed more than 10 years ago in Gaucher disease and MPS II, but did not show improvement of disease pathology, probably due to low levels of expression of the inserted transgene^{121,122}.

Other clinical trials for LSDs involve the direct administration of the transgene by the use of AAV vectors (table 4). The Phase/II clinical trial conducted for Pompe's disease is focused on the local delivery of the transgene with an AAV vector injected into the diaphragm¹²³. The investigators observe a significant improvement in respiratory function and no adverse effects have been reported so far¹²⁴.

Recently, the preliminary outcomes of two lentiviral gene therapy clinical trials were published^{125,126}. The gene therapy trial for the neurodegenerative disorder metachromatic leukodystrophy, involved 3 patients. HSCs of these patients were genetically modified with lentiviral vectors to introduce a functional gene and were the monitored for almost 2 years. Sustained engraftment of the modified HSCs was achieved, which led to a high expression of the introduced genes. The same set-up was used in the gene therapy trial to

Table 4: Overview of clinical trials for LSDs, as in the NCT database. Note: Clinical trials for MLD and WAS are not included in the NCT database.

Storage disorder	Trial start date	Follow up	Vector	Gene therapy clinical trial
Batten	2004	18 months	AAV	NCT00151216, NCT01414985,
	2010	18 months	AAV	NCT01161576
Fabry	1999		RV	NCT00001234,
Gaucher	1988			NCT00001234,
	1999	Life-long	RV	NCT00004294
Mucopolysaccharidosis I, II	1999	Life -long	RV	NCT00004454
Mucopolysaccharidosis III, IV	2011	1 year	AAV	NCT01474343
Pompe	2010	1 year	AAV	NCT00976352

treat 3 patients with Wiskott-Aldrich syndrome. At time of the report, the patients were monitored for over 2 years and sustained engraftment and expression of the introduced gene were achieved. The researchers in both studies looked at the integration patterns of the lentiviral vectors used, and there was no evidence of insertion of the integrating vectors near proto-oncogenes. However, further follow-up is required. These reports are important for the progress of the field of gene therapy.

Alternative approaches to genetic modifications

Integration of foreign DNA into the host genome by viral vectors is considered to be a risk. In fact, any integration into the genome can alter the expression of genes near the insertion site. To minimize the risk of insertional mutagenesis, lentiviral vectors are considered favorable due to their fairly random integration pattern. Other than the retroviral vectors they do not show a preference for transcriptional start sites. However, even when these vectors integrate stochastically within the host genome, the risk of vector insertion

related oncogenesis is present. Precise repair or replacement of non-functional genes is the ultimate goal of gene therapy. Several studies have shown promising results. For instance, cleavage of the host DNA at pre-selected sites followed by foreign DNA insertion via homologous recombination can be achieved using Zinc-finger nucleases; these are artificially made endonucleases with a FokI cleavage domain and a Cys2His2 Zinc-finger. Despite the progress in this field, therapies based on Zinc-finger technology are still far away from clinical application, mostly due to the Zinc-finger related genotoxicity and the quantification of this event. Off-target cleavage is still a major concern. Recently, new chimeric nucleases other than Zinc-finger nucleases have emerged, so called Transcription Activator-Like Effector Nucleases (TALENs)¹²⁷. TALEN-binding sites can be designed every 35 base pairs, and this would be an advantage over the Zinc-finger technology to insert DNA at pre-selected sites. Another advantage of the TALEN technology is that the protocols are rather simple.

However, up till now no one has succeeded in highly efficient gene targeting in HSC, (up to 13% efficiency so far) using Zinc-finger or TALENs technology. For some disease models 13% efficiency can be sufficient, but for others, like Pompe's disease, a higher expression of the transgene in the transplanted HSC is needed.

AIMS AND OUTLINE OF THIS THESIS:

This thesis deals with the development of lentiviral gene therapy mediated by hematopoietic stem cells for Pompe's disease in the relevant mouse model.

The aims of the thesis are:

- To demonstrate the potential of *ex vivo*, hematopoietic stem cells (HSCs) mediated, lentiviral gene therapy for the treatment of Pompe's disease in a murine model;
- to investigate whether complete correction of the Pompe's disease phenotype is possible;
- to determine the impact of age at therapeutic intervention on the therapeutic outcome at one integration of *GAA* per genome.

The outline of the thesis is as follows: Chapter 2 illustrates the rationale of using hematopoietic stem cell mediated lentiviral gene therapy in Pompe's disease: amelioration of the disease phenotype is achieved in a mouse model of Pompe's disease using a lentiviral vector with a strong viral promoter. Chapter 3 describes the application of codon optimization as a means to enhance the effect of hematopoietic stem cell mediated lentiviral gene therapy. The effect of gene therapy with a codon-optimized acid alpha-glucosidase gene construct (*GAAco*) significantly improved relative to the unmodified construct (*GAA*) in that all the accumulated glycogen is cleared in all tissues in the Pompe's mouse model. Chapter

4 describes the impact of age at time of treatment on the effect of treatment: lentiviral mediated gene therapy via HSCs poses challenges in both old mice and in young mice with one integration per genome. Chapter 5 reviews the options and challenges with regard to *ex vivo* expansion of muscle stem cells and their potential for treatment of muscular dystrophies. Chapter 6 summarizes the studies described in this thesis and discusses the outcome in a broader perspective. This last chapter also presents strategic considerations on translation of preclinical work into a registered gene therapy product.

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Stem cell based gene therapy

for Pompe's disease

acid alpha-glucosidase

Integrations

Hematopoietic stem cells

Muscle

Mouse

Single intervention

Lentivirus

Future

Storage

ERT

Transplantation

Costs

Glycogen

Immune tolerance

Transplantation

ABSTRACT

Pompe's disease (acid α -glucosidase deficiency) is a lysosomal glycogen storage disorder characterized in its most severe early-onset form by rapidly progressive muscle weakness and mortality within the first year of life due to cardiac and respiratory failure. Enzyme replacement therapy prolongs the life of affected infants and supports the condition of older children and adults but entails life-long treatment and can be counteracted by immune responses to the recombinant enzyme. We have explored the potential of lentiviral vector mediated expression of human acid α -glucosidase in hematopoietic stem cells (HSC) in a Pompe mouse model. After mild conditioning, transplantation of genetically engineered HSC resulted in stable chimerism of ~35% hematopoietic cells that over-express acid α -glucosidase and in major clearance of glycogen in heart, diaphragm, spleen and liver. Cardiac remodeling was reversed and respiratory function, skeletal muscle strength and motor performance improved. Over expression of acid α -glucosidase did not affect overall hematopoietic cell function and led to immune tolerance to the protein as shown by challenge with the human recombinant protein. Based on the prominent and sustained therapeutic efficacy without adverse events in mice we conclude that *ex vivo* HSC gene therapy is a treatment option worthwhile to pursue.

INTRODUCTION

Glycogenosis type II (GSDII, Pompe's disease, acid maltase deficiency; Online Mendelian Inheritance in Man [OMIM] # 232300) is an autosomal recessive lysosomal storage disorder caused by acid α -glucosidase (GAA) deficiency. The disease is characterized by glycogen storage in liver, spleen, kidney, brain, and endothelial cells, and most prominently in skeletal, heart and smooth muscles. Symptoms arise from muscular weakness and wasting. Infants with complete enzyme deficiency present shortly after birth, lose all muscle strength within eight months, and succumb to hypertrophic cardiomyopathy and respiratory failure in the first year of life.¹ Children and adults with residual acid α -glucosidase activity show a more protracted course and may become wheel chair bound, dependent on artificial ventilation and have a shortened life expectancy. Presently, enzyme replacement therapy (ERT) based on intravenous infusion of recombinant human α -glucosidase, taken up by mannose 6-phosphate receptor mediated endocytosis,^{2,3} is a major therapeutic advance that prolongs the life of affected infants, but does not guarantee long-term symptom free survival, requires biweekly administration and may induce immune responses to the recombinant protein.

As an alternative to ERT, *in vivo* gene therapy mediated by adenoviral vectors and adeno-associated virus vectors (AAV) has been investigated in a mouse model of Pompe's disease.⁴⁻⁶ However, long term efficacy can be significantly hampered by antibody formation,^{7,8} and adverse immune responses to the vector has been observed after adenoviral and AAV gene therapy in patients.^{9,10}

For treatment of patients with other lysosomal enzyme deficiencies, allogeneic hematopoietic stem cell (HSC) transplantation has been proposed.¹¹ HSC transplantation proved effective in ameliorating the neurological symptoms in murine globoid cell leukodystrophy (GLD) and human patients^{12,13} as well as in ameliorating mucopolysaccharidosis I (MPSI, Hurler syndrome).^{14,15} Other lysosomal storage disorders such as metachromatic leukodystrophy (MLD) may require higher enzyme levels than provided by HSC transplantation; lentiviral (LV) vector mediated overexpression of aryl-sulfatase A (ARSA) in HSCs effectively reversed the neuropathological phenotype in the mouse model¹⁶. In addition, lentiviral-mediated clinical gene therapy in trial phase of X-linked adrenoleukodystrophy halted progressive cerebral demyelination in two patients.¹⁷ Recently, HSC transplantation was demonstrated to promote immune tolerance to ERT in the Pompe mouse model.¹⁸ The use of gene modified autologous HSC also overcomes the profound conditioning and immune barriers associated with allogeneic transplantation.

The few attempts of HSC transplantation for Pompe's disease have not met with success.¹⁹ Acid α -glucosidase levels, if any, are low in hematopoietic cells in mice¹⁸ and allogeneic transplantation is not an obvious treatment. Therefore, high-level vector driven ectopic expression of the enzyme in hematopoietic cells would be required to accomplish

efficacy. We tested the hypothesis that *ex vivo* LV vector mediated over expression of acid α -glucosidase in a relatively small number of HSC would be beneficial following transplantation in preventing or reversing clinical symptoms of Pompe's disease, and deliberately chose a competitive repopulation strategy, in which the transduced stem cells compete with residual endogenous and non-transduced cells in the transplant, resulting in stable partial chimerism with the main hematopoietic system untouched by genetic modification.

MATERIALS AND METHODS

Animals

Normal FVB and congenic *Gaa*^{-/-} knock out mice were obtained as described.²⁰ The animals do not produce *Gaa* mRNA, have a complete deficiency of acid α -glucosidase and parallel the human infantile-onset Pompe's disease by pathological criteria and clinical symptoms, (i.e. heart and skeletal muscle weakness.)^{20,21} The animal experiments were reviewed and approved by an ethical committee consistent with legislation in The Netherlands.

Cell culture

Mesenchymal stem cells (MSCs) for titration of LV vectors were isolated from bone marrow (BM) of *Gaa*^{-/-} mice by flushing femurs. MSCs were allowed to attach overnight in 10 cm² tissue culture dishes and the remaining hematopoietic cells were removed by washing. MSCs were cultured in a mixture of 48% Dulbecco's Modified Eagle's Medium with low glucose (DMEM-LG, Gibco), 32% MCDB 201 medium (Sigma) and 20% fetal bovine serum (FBS, Gibco) with addition of penicillin and streptomycin (pen/strep, Gibco). Human embryonic kidney (HEK) 293T cells were cultured in DMEM medium supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine (Sigma) and penicillin and streptomycin.

Construction of lentiviral vector plasmids

The human *GAA* (hGAA) cDNA was excised with EcoRI and SphI from plasmid pSHAG2²² and cloned in the pSUPER vector (Oligoengine) containing a polylinker with sequential AgeI, EcoRI, SphI and XbaI restriction sites (pSli). The pSli-GAA plasmid was then digested by AgeI, XbaI and subcloned in a LV vector backbone with sequential AgeI, NheI, SwaI restriction sites. The final plasmid was constructed by removal of the hGAA cDNA containing AgeI-SwaI fragment to replace EGFP of pRRL.PPT.SF.EGFP.WPRE4*.SIN (LV-SF-GFP, kindly provided by Axel Schambach, Hannover Medical School, Germany) to obtain the LV vector LV-SF-GAA. The vectors contain the HIV central polypurine tract and the spleen focus forming virus (SF) promoter to drive transgene expression as well as modified woodchuck posttranslational regulatory element (WPRE4*) with 4 deleted ATG sites and a large deletion of the Woodchuck hepatitis X-protein sequence.²³

Production of lentiviral vectors

Third-generation self-inactivating LV vectors were produced by standard calcium phosphate transfection of HEK 293T cells with the plasmids pMDL-g/pRRE, pMD2-VSVg and pRSV-Rev.^{24,25} Titers of LV-SF-GFP were determined by end-point titration on mouse *Gaa*^{-/-} MSCs by flow cytometry. LV-SF-GAA vector titers were determined by counting immunofluorescence stained MSCs (Supplemental figure 1). For *in vivo* studies LV vectors were concentrated by ultracentrifugation (2 hours, 20,000 r.p.m., 4°C) and stored until use.

Lentiviral hematopoietic stem cell transduction

Donor BM was extracted from femurs and tibias of eight to twelve week-old male *Gaa*^{-/-} mice, and hematopoietic progenitors were purified by lineage depletion (Lin⁻) according to the manufacturer's protocol (BD). After enrichment, Lin⁻ cells were transduced by LV-SF-GFP or LV-SF-GAA LV vectors overnight at a cell density of 10⁶ cells/mL and a multiplicity of infection (MOI) of 9-10 in serum free modified Dulbecco's medium as described²⁶, supplemented with growth factors (murine-Stem Cell Factor 100ng/ml, human FMS-like tyrosine kinase 3 murine, 50ng/ml, mouse Ligand thrombopoietin 10ng/ml). The following day, 5x10⁵ transduced Lin⁻ cells were injected in the tail vein of eight to twelve week-old female *Gaa*^{-/-} recipients subjected to a sublethal total body radiation dose of 6 Gy.

Tissue preparation

At termination of experiments, mice were fasted overnight, anesthetized with isoflurane and sacrificed by transcardial perfusion with phosphate buffered saline (PBS). The following tissues were collected: heart, diaphragm, stomach, uterus, quadriceps femoris (QF), spleen, lung, liver, kidney and brain. For assay of GAA activity, tissue aliquots were snap frozen in liquid nitrogen and stored at -80°C. Tissue aliquots for Periodic Acid Schiff (PAS) staining were fixed in 1% formaldehyde + 4% glutaraldehyde solution in 0.1% cacodylate, pH 7.3. PAS staining was performed as described²⁰ and tissue slides were quantified using ImageJ (National Institutes of Health).

Immunofluorescence staining

MSCs plated on glass cover slips were LV-SF-GFP or LV-SF-GAA transduced and fixed 5 days later with ice-cold methanol/acetone (4:1) for 10 minutes. Similarly, BM cells were allowed to attach onto Retronectin® (Takara Inc) coated cover slips for 4 hours and fixed. The cells were blocked by incubation in 10% FCS in PBS with 0.05% Tween for 30 minutes and subsequently incubated with anti-GAA antibody, raised in rabbits against human placental enzyme, but also recognizes the mouse enzyme²⁰ at 1: 100 dilution in blocking solution (2% FCS/PBS/0.05% Tween), followed by 1 hour with goat anti-rabbit-Alexafluor 488 (1:500, Invitrogen). Finally, stained cells were embedded in Vectashield mounting medium (Vector Laboratories) containing DAPI (4'-6'-diamidino-2-phenylindole). The slides were

examined under a fluorescence microscope (Leica DMRXA) with attached camera (Leica DFC 350FX) and analyzed with Leica FW4000 Version 1.2.1.

Enzymatic assays

All tissues were homogenized in water by sonication (MSE sonifier) on ice until completely lysed (medium level, amplitude 5 μ m). The GAA activity was determined by fluorometry, according to a protocol based on 4-methylumbelliferyl α -D-glucopyranoside (Sigma-Aldrich).²⁷ Plasma was collected in lithium heparin coated blood-sampling tubes (BD Microtainer) with gel and was centrifuged immediately after collection to separate plasma from cells. GAA was extracted from the plasma samples with anti-GAA antibodies in combination with protein A-sepharose beads before to measurement of enzyme activity to avoid non-specific the activity of nonspecific glycosidases.

Glycogen content was determined as described². The resulting glucose was measured after conversion by glucose-oxidase and reaction with 2,2'-azino-di-(ethyl-benzthiazolin-sulfonate) (ABTS). The GAA activity and the glycogen content were both calculated on the basis of protein content of the homogenates, which was measured with the use of the bicinchoninic acid protein assay (Pierce).

Western blotting

Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose filters, incubated with anti- α -glucosidase antiserum (1250 \times diluted) and the complex of enzyme and antibody was visualized with an Odyssey Infrared Imaging System (Li-Cor Biosciences) with the use of goat anti-rabbit immunoglobulin G (IgG) conjugated to IRDye 800 CW (1:10 000) as secondary antibody.

Immunization of mice and anti-human acid α -glucosidase ELISA

Wild-type (WT), *Gaa*^{-/-} LV-SF-GFP⁻ and LV-SF-GAA⁻ treated mice were injected with human recombinant GAA [Myozyme (alglucosidase alfa); 20 mg/kg] together with Freund adjuvant and 2 weeks later rechallenged with human recombinant GAA. Plasma was collected before and 2 weeks after each injection.

For anti-human GAA enzyme-linked immunoabsorbent assay (ELISA), human GAA and GFP were produced in HEK 293T cells by LV transduction, confirmed by enzymatic assay and GFP by fluorescence microscopy, respectively. ELISA plates (Nunc) were coated overnight with 5 μ g/mL cellular lysate in PBS, pH 7.4, blocked with 1% bovine serum albumin in PBS, washed, and incubated with serial dilutions of plasma from WT, LV-SF-GFP⁻, or LV-SF-GAA⁻ treated mice. After washing, mouse immunoglobulins were detected with anti-mouse IgG peroxidase (Sigma- Aldrich) and 3,3', 5,5'-tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories). ELISAs were performed in triplicate with the same plasma samples applied to GAA- and GFP-coated plates.

Quantitative PCR of lentiviral integrations

The DNA Nucleospin kit (Bioke) was used to extract DNA from BM and spleen cells to determine the copy number per cell by quantitative polymerase chain reaction (q-PCR) in an ABI PRISM 7900 HT sequence detection system (Applied Biosystems).²⁸ Reactions were performed with 50 ng of genomic DNA with SYBR Green PCR Master Mix (Applied Biosystems) with the following primer set: HIV-U3 forward primer, 5'-CTGGAAGGGC-TAATCACTC-3', and HIV reverse primer, 5'-GGTTTC- CTTTTCGCTTTCAG-3'. To assess chimerism, primers specific for the *Sry* locus on the mouse Y chromosome were developed: forward primer, 5'-CATCG- GAGGGCTAAAGTGTCAC-3', and reverse primer, 5'-TG-GCATGTGGGTTCC- CTGTCC-3'. The samples were normalized for mouse *Gapdh* with the following primer set: forward primer, 5'-CATCACTGCCACCCAGAAGAC-3', and reverse primer, 5'-TGACCTTGCCACAGCCTTG-3'. A standard line for the LV integrations was determined from sorted mouse 3T3 cells transduced at a multiplicity of infection of 0.06. Samples were analyzed with SDS2.2.2 software (Applied Biosystems).

Echography and hemodynamics

Mice were sedated with 4% isoflurane, intubated,²⁹ and ventilated with a mixture of O₂ and N₂O (1/2; vol/vol) with a pressure-controlled ventilator (CWE, SAR-830/P) to which 2.5% isoflurane was added for anesthesia. Ventilation rate was set at 90 strokes/min with a peak inspiration pressure of 18 cm H₂O and a positive end expiration pressure of 4 cm H₂O. The mice were placed on a heating pad to maintain body temperature at 37°C.

In vivo transthoracic echocardiography of the left ventricle was performed with the ALOKA echo device (Pro Sound, SSD-4000) with a 13-MHz linear interfaced array transducer.²⁹ M-mode echocardiograms were captured from short-axis 2-dimensional views of the left ventricle at midpapillary level with simultaneous electrocardiogram. Left ventricular lumen diameters at end diastole and end systole as well as wall thickness were measured from the M-mode images with the use of SigmaScan Pro 5 Image Analysis software (SPSS Inc). Three cardiac cycles for each animal were analyzed by a blinded observer. Fractional shortening was calculated with the following equation: [(ventricular lumen diameter at end diastole - ventricular lumen diameter at end systole)/ventricular lumen diameter at end diastole x 100%]. After echocardiography, a 1.4F Millar Instruments pressure transducer catheter (SPR-671; Millar Instruments; calibrated before each experiment with a mercury manometer) was inserted in the right carotid artery and advanced into the left ventricle for measuring left ventricular pressure and heart rate. From the left ventricular pressure signal, we obtained the rate of rise of left ventricular pressure at left ventricular pressure of 30 mm Hg (dp/dtP30), as a measure of left ventricular systolic function, the time constant of left ventricular relaxation (Tau), as well as the left ventricular end-diastolic pressure were obtained as parameters of diastolic function.²⁹ At the conclusion of each experiment, the heart was excised, the atria were removed, and the right ventricle and left ventricle (including septum) were separated and mass determined.

Hemodynamic data were recorded and digitized with an online 4-channel data acquisition program (ATCODAS; Dataq Instruments) for later analysis with a program written in MatLab (MathWorks). A minimum of 10 consecutive beats were selected for analysis.

Ventilatory function

Airway function was tested by whole-body plethysmography (Buxco Electronics) comparable with a setting used to assess airway hyperresponsiveness in patients with asthma to metacholine. This test can be influenced by muscular weakness and exhaustion. Mice were exposed to nebulized physiologic saline for 3 minutes and then to increasing concentrations of nebulized metacholine in PBS (1.56-50mg/mL) with the use of an ultrasonic nebulizer. Measurements were obtained for 3 minutes after the completion of each nebulization. The following parameters were measured: the average peak inspiratory flow (PIF; mL/sec), tidal volume (TV; mL/ breath), and frequency of breathing (breaths/min).

Motor performance

To determine skeletal muscle strength, grip measurement was performed with the Bioseb Grip Strength test. The mice were held by the tail and pulled backward over the grid with all limbs to measure the magnitude of the peak force (in mN). Three single pulls per mouse were averaged. All data points were collected over time in mice older than 150 days, and averages per mouse were used to compare treatment groups.

Motor muscle tasks were performed with the accelerating rotarod (from 4 to 40 rpm in 5 minutes; model 7650, Ugo Basile Biologic Research apparatus). Mice were given 2 trials to adjust to the apparatus, followed by 3 runs with intervals of 5 minutes, which were averaged.

The running performance was measured by a computerized system connected to running wheels.³⁰ The distance run was calculated by multiplying the revolutions with the circumference of the wheels.

Peripheral blood counts, phenotyping and *in vitro* clonogenic progenitor assays

Blood cell counts were measured using a Vet ABC hematology analyzer (Scil animal care company GmbH, Germany). Peripheral blood, BM and spleen cells were stained with antibodies against CD3, CD4, CD8, B220, IgM, CD11b, Gr-1, Sca-1, c-Kit directly conjugated to PE or APC (All BD Biosciences) and measured by flow cytometry. *In vitro* clonogenic assays were performed as described.^{26,31-34}

Statistical analysis

Statistical analysis was performed with SPSS 11 (SPSS Inc). Significance of differences was determined by 1-way analysis of variance or Mann-Whitney *U* test for comparing 2 groups. For repeated measurements 2-way analysis of variance was used to determine differences between groups (for *P* values, see supplemental data). Significance of a difference was assumed if the *P* value was less than .05. All error bars represent SEM.

RESULTS

Ex vivo transduction of BM cells leads to high levels of acid α -glucosidase activity in *Gaa*^{-/-} mice

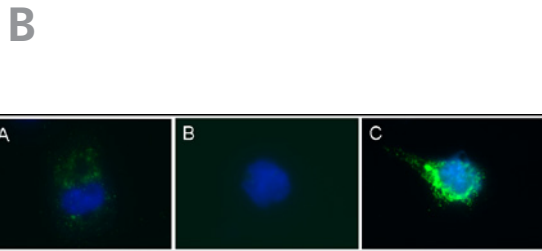
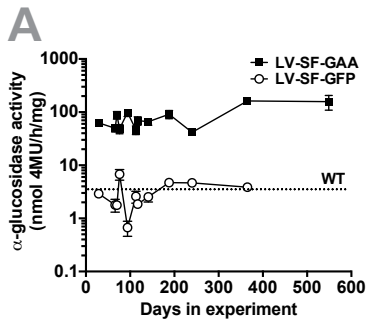
Gaa^{-/-} recipients of LV-SF-GFP transduced Lin⁻ male BM cells displayed long-term expression of GFP in blood as measured by flow cytometry (data not shown). LV-SF-GAA-treated mice displayed high levels of GAA activity in leukocytes (average of all measured samples is 71.1 ± 47.3 nmol/h/mg protein, $n=77$, Figure 1A) for a observation time of 1.5 year, 35-fold higher than background values measured in LV-SF-GFP mice (2.0 ± 1.7 nmol/h/mg protein, $n=81$) and also higher than in 8 months old WT mice (Table 1, Figure 1A). GAA activity at eight months in WT mice was not different from background levels detected in *Gaa*^{-/-} mice. This shows that GAA levels in leukocytes are hardly detectable (Figure 1A; Table1).

High GAA activity was also detected in BM and spleen cells of LV-SF-GAA treated mice 8 months and 1.5 year after treatment (Table 1), respectively, with levels in WT mice being approximately 2.5% of levels in LV-SF-GAA treated mice. GAA protein in BM cells was confirmed by immunofluorescence staining in LV-SF-GAA-treated mice (Figure 1B). In addition, molecular species of intermediate (95 kDa) and mature (76 kDa) forms of GAA were detected by Western blotting after LV-SF-GAA-treatment at 10-months of age (Figure 1C). GAA expression in WT mice could not be detected by Western blotting consistent with the low levels of activity in WT leukocytes, but was clearly enhanced in plasma of LV-SF-GAA mice (Figure 1D)

The long-term expression of GAA in hematopoietic cells resulted in reconstitution of activity in target tissues (Figure 1E), that is, heart, smooth muscle containing tissues (diaphragm, stomach and uterus), in skeletal muscle measured in the QF and in lung and liver, but not in brain tissue. The data show that sublethal irradiation and transplantation of a clinically feasible number of hematopoietic cells results in stable ectopic production and high levels of human α -glucosidase in affected target tissues. Notably, challenging the mice with the recombinant human enzyme with Freund adjuvant showed that expression in hematopoietic cells also resulted in immune tolerance to the transgene product (Figure 1F).

Table 1: Acid α -glucosidase activity in bone marrow and spleen of mice that received a transplant of LV-SF-GAA. Activity displayed as α -glucosidase activity (nmol/h/mg of protein) \pm SD. Values of LV-SF-GFP are background glycosidase levels.

Group	Leukocytes	Bone marrow	Spleen
WT (8 mo)	3.3 ± 2.3 ($n = 6$)	3.2 ± 2.6 ($n = 6$)	5.2 ± 3.6 ($n = 12$)
LV-SF-GFP (8 mo)	4.7 ± 1.3 ($n = 6$)	0.5 ± 0.5 ($n = 6$)	0.6 ± 0.6 ($n = 15$)
LV-SF-GAA (8 mo)	42 ± 7 ($n = 6$)	107 ± 43 ($n = 5$)	249 ± 280 ($n = 5$)
LV-SF-GAA (18 mo)	157 ± 97 ($n = 4$)	132 ± 69 ($n = 4$)	162 ± 111 ($n = 4$)



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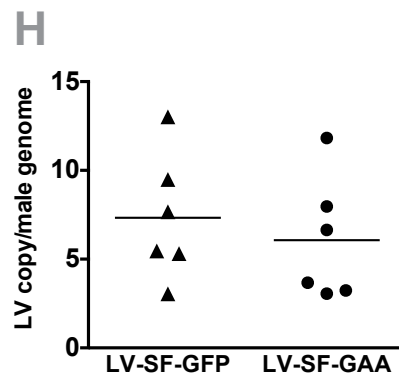
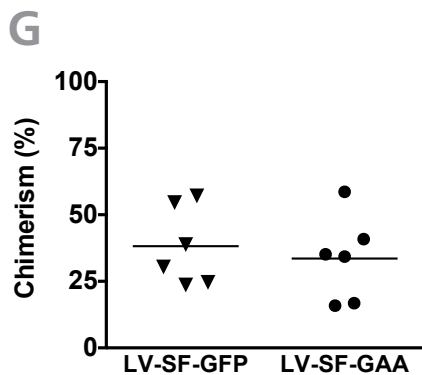
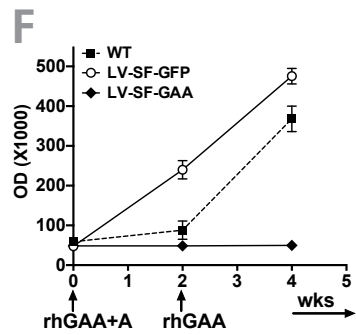
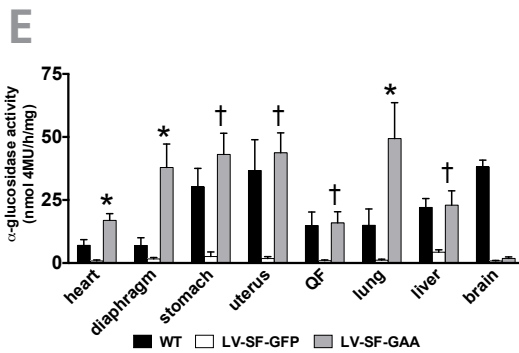
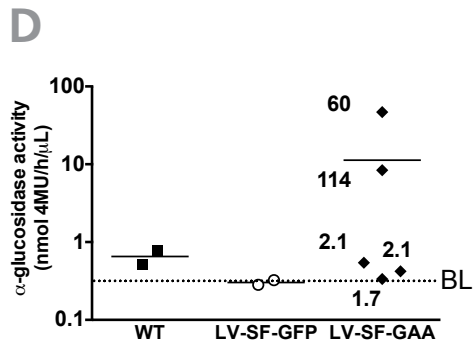
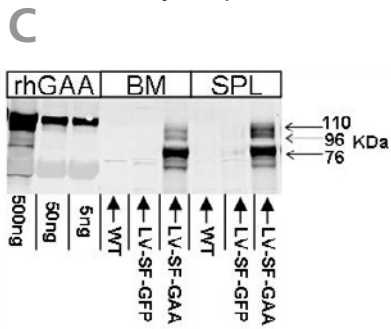


Figure 1. In vivo lentiviral expression of human acid α -glucosidase.

(A) *Gaa*^{-/-} mice were transplanted with 5×10^5 Lin⁻ cells transduced with the therapeutic vector (LV-SF-GAA) at a MOI of 9-10. The acid α -glucosidase activity in peripheral leukocytes of a total of 32 LV-SF-GAA treated mice (closed squares) was measured starting from day 29 after transplantation to the last data point at day 549 (n=4). Fifteen LV-SF-GFP transplanted *Gaa*^{-/-} mice (open circles) served as negative controls. The leukocyte α -glucosidase levels of congenic FVB mice (n=6) are indicated by the dotted line (3.4 ± 1.3 nmol/h/mg).

(B) Illustration of acid α -glucosidase expression and lysosomal localization in BM cells of a WT, and a *Gaa*^{-/-} mouse, and a mouse transplanted with LV-SF-GAA transduced HSCs harvested 8 months after transplantation (blue nuclear DAPI stain and green fluorescent Alexafluor-488 immunostaining for acid α -glucosidase protein, original magnification $\times 1000$).

(C) At 8 months after transplantation, Western blot analysis of acid α -glucosidase expression in bone marrow (BM) and spleen (SPL) reveals a known pattern of 95kDa (intermediate species) and 76kDa (mature species) forms of human acid α -glucosidase (G) reflecting acid α -glucosidase synthesis, post translational modification, maturation, and lysosomal localization²². LV-SF-GFP transplanted mice (K) do not express human acid α -glucosidase, while the level of acid α -glucosidase expression in wild type animals (W) is too low to detect. A dilution of recombinant human acid α -glucosidase (rhGAA) of 110 kDa serves as a marker.

(D) Plasma acid α -glucosidase enzyme levels. LV-SF-GAA transplanted *Gaa*^{-/-} mice showed variable, but detectable acid α -glucosidase enzyme levels in plasma compared to background activity (BL) detected in *Gaa*^{-/-} mice. Low activity levels were also detected in WT mice. Numbers in the figure that accompany the plasma data represent acid α -glucosidase activity in leukocytes (nmol/h/mg).

(E) Reconstitution of acid α -glucosidase activity in *Gaa*^{-/-} mice. Acid α -glucosidase activity in tissues of LV-SF-GFP and LV-SF-GAA transplanted, and age matched WT mice at 10 months of age (n=3 to 6 mice for all tissues, except for LV-SF-GFP brain; * indicates a significant difference ($P < 0.05$) of LV-SF-GAA transplanted mice compared to both WT and LV-SF-GFP mice. † indicates a significant difference ($P < 0.05$) between LV-SF-GAA and LV-SF-GFP transplanted mice, but between LV-SF-GAA and WT mice.

(F) Tolerance to recombinant human acid α -glucosidase (rhGAA) in LV-SF-GAA transplanted *Gaa*^{-/-} mice. Both WT (n=2) and LV-SF-GFP treated *Gaa*^{-/-} mice (n=2) responded by the generation of anti-human acid α -glucosidase antibodies after a first injection of recombinant human acid α -glucosidase (Myozyme®, 20mg/kg) with Freund's complete adjuvant (rhGAA+A) followed by a second injection of recombinant human acid α -glucosidase without Freund's adjuvant (rhGAA) two weeks later. LV-SF-GAA treated *Gaa*^{-/-} mice (n=13) did not respond to the challenge with recombinant human acid α -glucosidase. BL = baseline OD.

(G) The percentage of reconstituted male cells in BM of LV-SF-GFP and LV-SF-GAA female recipients (chimerism) was determined by Y-chromosome *Sry* Q-PCR and corrected for overall genomic DNA by *Gapdh* Q-PCR (n=6).

(H) Copy number of LV vector integrations. Q-PCR was used to determine the average copy number in male BM cells (n=6).

Hematopoietic chimerism and transgene copy number per cell

Chimerism, defined as the contribution of transplanted male cells to overall blood cell production in BM, is shown in figure 1G. The percentage of Y-chromosome positive BM cells was on average 38 ± 15 for LV-SF-GFP treated *Gaa*^{-/-} mice (N=6) and 34 ± 16 for LV-SF-GAA treated mice (N=6). The percentage of GFP+ cells was 36 ± 12 in BM, and similar levels were observed in spleen and PB (N=7), consistent with the sub-ablative regimen used. Transduction efficiencies by measurement of GFP expression *in vitro* were greater than 70%; thus most transplanted donor cells contained the integrated LV vector.

Q-PCR was performed to determine the LV copy number per cell in BM of LV-SF-GFP and LV-SF-GAA-treated mice (Figure 1H). The percentage of chimerism in BM of LV-SF-GFP and LV-SF-GAA mice was used to calculate the number of integrations per transduced cell (LV

vector copies/fraction male BM genomes of total BM genomic DNA). LV-SF-GAA-treated mice were corrected with the same formula, which resulted in similar copy numbers per cell in LV-SF-GAA and LV-SF-GFP treated mice of 7.3 ± 3.6 and 6.1 ± 3.5 respectively.

Transplantation of acid α -glucosidase expressing HSC reduces glycogen deposition

To evaluate the effect of over expressed acid α -glucosidase, we examined the tissues by histology and quantitative PAS staining. LV-SF-GAA-treated *Gaa*^{-/-} mice had a significant reduction in glycogen storage compared to LV-SF-GFP treated *Gaa*^{-/-} mice in various tissues, ie, heart, diaphragm, liver and spleen (Figure 2). Importantly, glycogen assay confirmed the robust glycogen reduction in heart of LV-SF-GAA-treated mice, as well as in lungs and liver, and in tissues that contained smooth muscle, such as diaphragm, stomach and uterus, with a limited reduction in QF, representing skeletal muscles, and none in brain tissue (both cortex and cerebellum).

Transplantation of GAA expressing HSCs reverses cardiac remodeling

Major differences in heart geometry between WT, LV-SF-GFP and LV-SF-GAA-treated mice are depicted in Figure 3. In agreement with previous observations,³⁵ *Gaa* deficiency in LV-SF-GFP mice results in an elevated mass of both the right and the left ventricle at 10 and 15 months relative to those in WT mice (Figure 3A,B), resulting from an increased wall thickness (Figure 3C, D), whereas left ventricular lumen diameter was not significantly affected (not shown). Mean aortic pressure was not significantly affected either (99 ± 3 mmHg vs 89 ± 4 mmHg), but heart rate was significantly reduced in LV-SF-GFP mice compared with WT mice ($p < 0.01$; Figure 3E). Global left ventricular pump function was still maintained in LV-SF-GFP mice compared with WT mice, as evidenced by normal levels of indexes of systolic function, including left ventricle $dP/dt_{p_{30}}$ (7380 ± 660 mmHg/s vs 7930 ± 990 mmHg/s), and fractional shortening ($46\% \pm 2\%$ vs $43\% \pm 1\%$), as well as near normal levels of indexes of diastolic function, including time constant of relaxation Tau (15 ± 5 millisecond vs 12 ± 3 millisecond) and left ventricular end-diastolic pressure (8 ± 3 mm Hg vs 6 ± 2 mm Hg), in LV-SF-GFP and WT mice, respectively at 10 months (data in parentheses) and 15 months of age (data not shown). These findings confirm previous observations that up to this age, *Gaa*^{-/-} mice do not exhibit overt left ventricle dysfunction.³⁵

After transplantation of the GAA-overexpressing cells, both relative right ventricular and left ventricular mass decreased in LV-SF-GAA-treated animals compared to LV-SF-GFP mice (Figure 3A-B), accompanied by a restoration of left ventricular wall thickness towards levels observed in WT mice (Figure 3D). Heart rate also returned towards the levels observed in WT mice (Figure 3E), whereas mean aortic pressure (108 ± 3 mm Hg), fractional shortening ($41\% \pm 1\%$), left ventricle $dP/dt_{p_{30}}$ (8110 ± 840 mm Hg/s), Tau (14 ± 4 ms), and left ventricular end-diastolic pressure (8 ± 2 mmHg) remained principally unaffected (not shown).

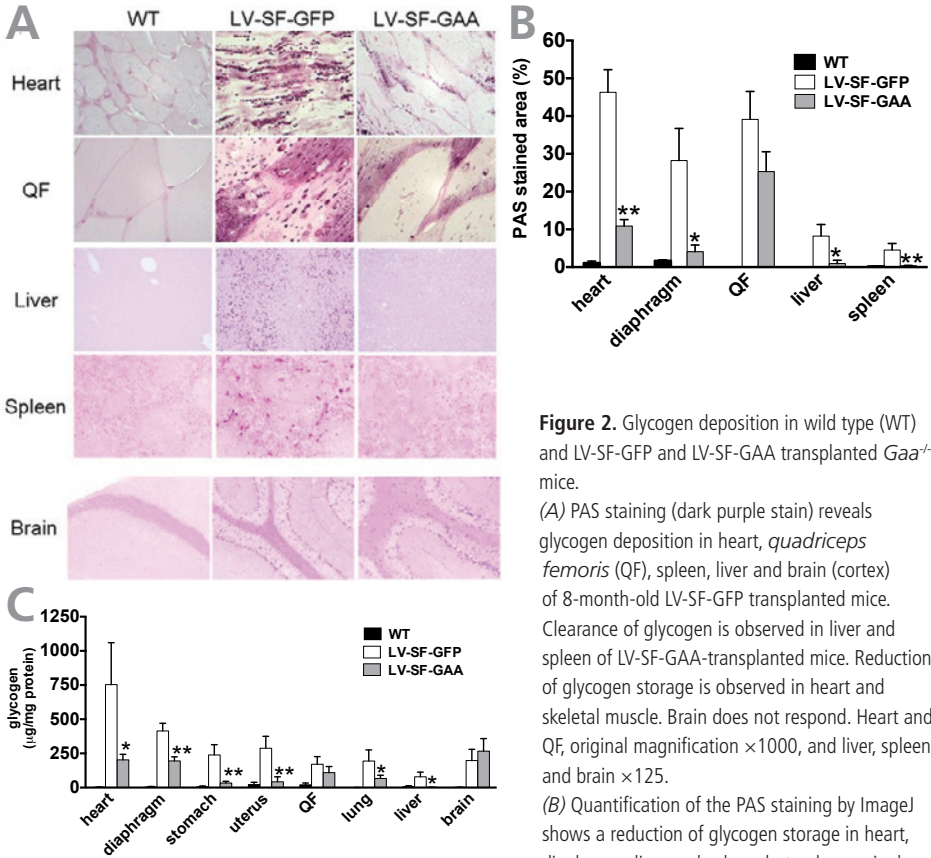


Figure 2. Glycogen deposition in wild type (WT) and LV-SF-GFP and LV-SF-GAA transplanted *Gaa*^{-/-} mice.

(A) PAS staining (dark purple stain) reveals glycogen deposition in heart, *quadriceps femoris* (QF), spleen, liver and brain (cortex) of 8-month-old LV-SF-GFP transplanted mice. Clearance of glycogen is observed in liver and spleen of LV-SF-GAA-transplanted mice. Reduction of glycogen storage is observed in heart and skeletal muscle. Brain does not respond. Heart and QF, original magnification $\times 1000$, and liver, spleen and brain $\times 125$.

(B) Quantification of the PAS staining by ImageJ shows a reduction of glycogen storage in heart, diaphragm, liver and spleen, but only marginal reduction in QF at 8 months after transplantation.

PAS stained area as % of total area, is presented for LV-SF-GFP and LV-SF-GAA groups $n=4$ to 6 (* $P<0.05$ and ** $P<0.01$ indicate a significant difference between LV-SF-GAA and LV-SF-GFP transplanted mice). (C) Enzymatic quantification of the glycogen deposition in tissues at 8 months after transplantation. The figure shows a reduction of glycogen in all tissues tested except brain. ($n=4$ to 11 , * $P<0.05$ and ** $P<0.01$ indicate a significant difference between LV-SF-GAA and LV-SF-GFP transplanted mice).

Transplantation of acid α -glucosidase expressing HSCs improves respiratory function

Gaa^{-/-} mice are known to have reduced respiratory function.⁵ Whole-body plethysmography was performed on WT, LV-SF-GFP and LV-SF-GAA-treated mice to test respiratory function (Figure 4). The parameters tested were peak inspiratory flow (PIF), tidal volume (TV) and breathing frequency. PIF, TV and breathing frequency were both significantly reduced in the LV-SF-GFP mice relative to the WT mice. The LV-SF-GAA-treated mice scored significantly better than the LV-SF-GFP mice in PIF and frequency of breathing (Figure 4).

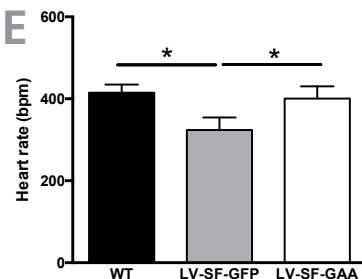
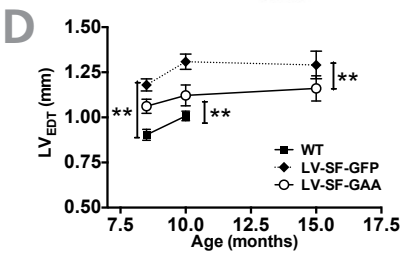
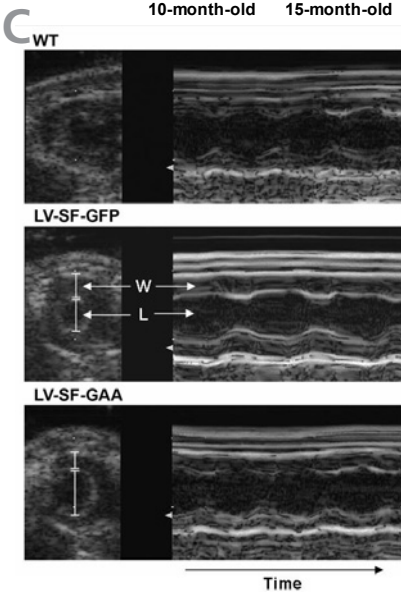
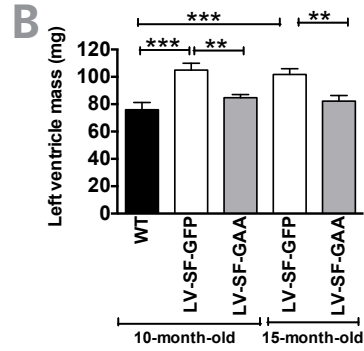
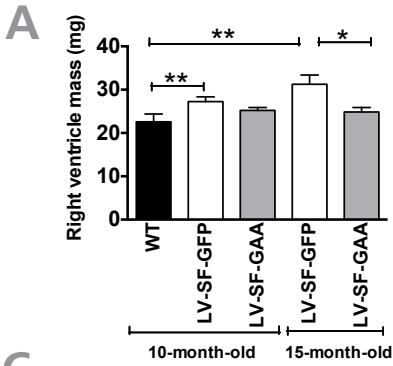


Figure 3. Effect of LV-SF-GAA HSC transplantation on cardiac morphology and parameters.

(A,B) Response of cardiac hypertrophy to LV-SF-GAA-transplantation. The right and left ventricular mass of 10-month-old ($n=6$ in all groups) and 15-month-old ($n=5$ for LV-SF-GFP and $n=7$ for LV-SF-GAA) LV-SF-GFP-transplanted mice is significantly higher than of WT mice. The left ventricular mass of LV-SF-GAA-transplanted mice is significantly lower than of the age matched LV-SF-GFP-transplanted mice. The same holds for the right ventricular mass at month 15 ($*P<0.05$, $**P<0.01$ or $***P<0.001$ indicate a significant difference between groups).

(C) Representative ultrasound images display the short axis of the left ventricle (2-D guided: left and M-mode: right) of WT, LV-SF-GFP and LV-SF-GAA-transplanted $Gaa^{-/-}$ mice (W = ventricular wall, L = left ventricular lumen). The LV-SF-GFP-transplanted $Gaa^{-/-}$ mice display a larger left ventricular wall thickness than the WT and LV-SF-GAA-transplanted mice, both at end-diastole (LV_{EDT}) and at end-systole (not shown). Furthermore, it shows that LV-SF-GFP-transplanted mice have a lower heart rate than both the WT and LV-SF-GAA-transplanted mice by the increased duration of the cardiac cycle. (D) The left ventricular wall thickness at end diastole (LV_{EDT}) in LV-SF-GFP treated mice ($n=6$) is increased compared to WT at 8 and 10 months of age ($n=6$). Reduction of LV_{EDT} is accomplished in LV-SF-GAA-transplanted mice compared to LV-SF-GFP treated mice at 8 ($n=6$), 10 ($n=6$) and 15 months of age ($n=3$) ($**P<0.01$). (E) Relative heart rate normalized after LV-SF-GAA treatment. At 10 months of age LV-SF-GFP treated mice ($n=10$) had a lower than normal (WT mice; $n=11$) heart rate. Treatment with LV-SF-GAA ($n=11$) normalized the heart rate ($**P<0.01$)

Transplantation of acid α -glucosidase expressing HSCs improves motor performance

Although the deposition of glycogen was less reduced in skeletal muscle than in other tissues, we tested the effect of treatment on muscle strength and motor performance of LV-SF-GAA-treated mice. As expected, the muscle strength of all paws of WT mice was significantly ($p < 0.001$) higher than that of LV-SF-GFP- (2.5-fold) and LV-SF-GAA-treated mice (2-fold) (Figure 5A). However, LV-SF-GAA treated mice were also significantly stronger (1.2-fold; $p = 0.002$) than LV-SF-GFP treated mice, showing efficacy on skeletal muscle strength. From the age of 6 months onward motor performance was tested by determining latencies on an accelerating rotarod and activity in running wheels. WT mice stayed significantly longer on the rod than LV-SF-GFP mice ($p < 0.05$). The difference between LV-SF-GAA treated and LV-SF-GFP-treated mice (Figure 5B) was not significant. Notably, three LV-SF-GFP-treated mice out of ten stayed less than 1 minute on the rod, which was not observed in the LV-SF-GAA-treated and WT mice. In the running wheels, WT mice performed best, and LV-SF-GAA-treated mice ran significantly ($p < 0.05$) longer distances than LV-SF-GFP-treated controls (Figure 5C).

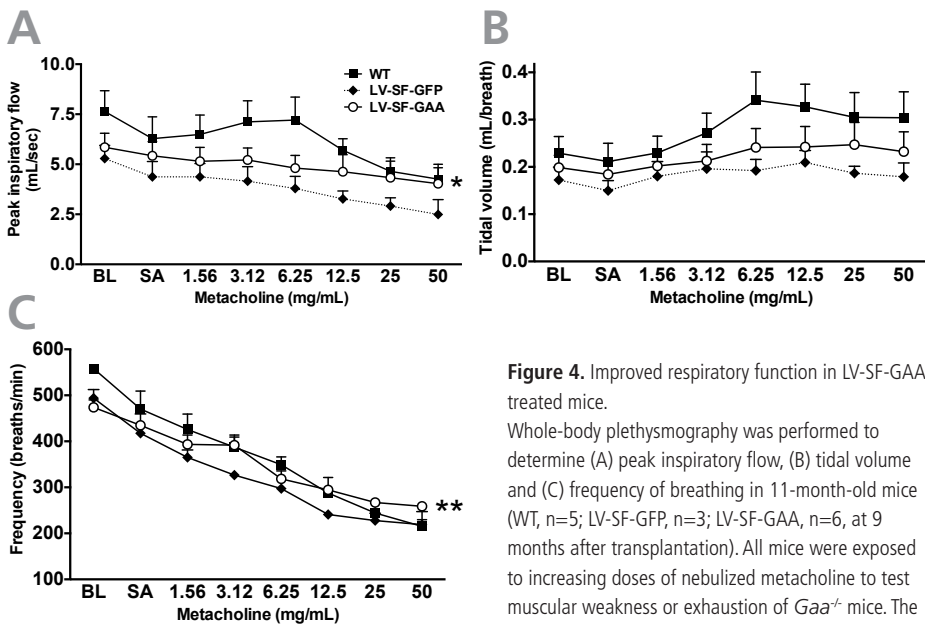


Figure 4. Improved respiratory function in LV-SF-GAA-treated mice.

Whole-body plethysmography was performed to determine (A) peak inspiratory flow, (B) tidal volume and (C) frequency of breathing in 11-month-old mice (WT, $n = 5$; LV-SF-GFP, $n = 3$; LV-SF-GAA, $n = 6$, at 9 months after transplantation). All mice were exposed to increasing doses of nebulized metacholine to test muscular weakness or exhaustion of *Gaa*^{-/-} mice. The peak inspiratory flow, tidal volume and frequency of breathing are reduced in LV-SF-GFP-transplanted

animals compared to WT mice. LV-SF-GAA transplantation improves both parameters compared to LV-SF-GFP (* $P < 0.05$, ** $P < 0.01$). BL, baseline; SA, saline.

High acid α -glucosidase expression in transplanted HSCs has no adverse effect on hematopoietic system function

High expression levels of GAA should not affect hematopoietic cell differentiation and function. White and red blood cell counts and parameters, as well as platelets (supplemental Table 4) did not show differences due to GAA overexpression. Phenotyping BM, spleen and peripheral blood (PB) cells showed that numbers of T lymphocytes (CD3, CD4, CD8), monocytes/granulocytes (CD11b, Gr1) and cells with stem-cell markers (Sca-1, c-Kit) did not differ significantly among WT, LV-SF-GFP and LV-SF-GAA-treated mice except for PB cells with B-lymphocyte markers (B220, IgM, CD19). B220+/IgM+ cells of LV-SF-GAA treated mice were increased (Supplementary Figure 2; 3.8 fold, $p < 0.01$) relative to the level of 188 ± 118 cells/ μ l in WT mice with a similar trend in LV-SF-GFP treated mice (2.4 fold, $p = 0.1$). This effect was not observed in BM or spleen and did not result in increased overall numbers of B-lymphocytes assuming a blood volume of 2 mL and one femur containing 8.5% of total mouse BM cells per femur.³⁶ Functional colony cultures of BM and spleen

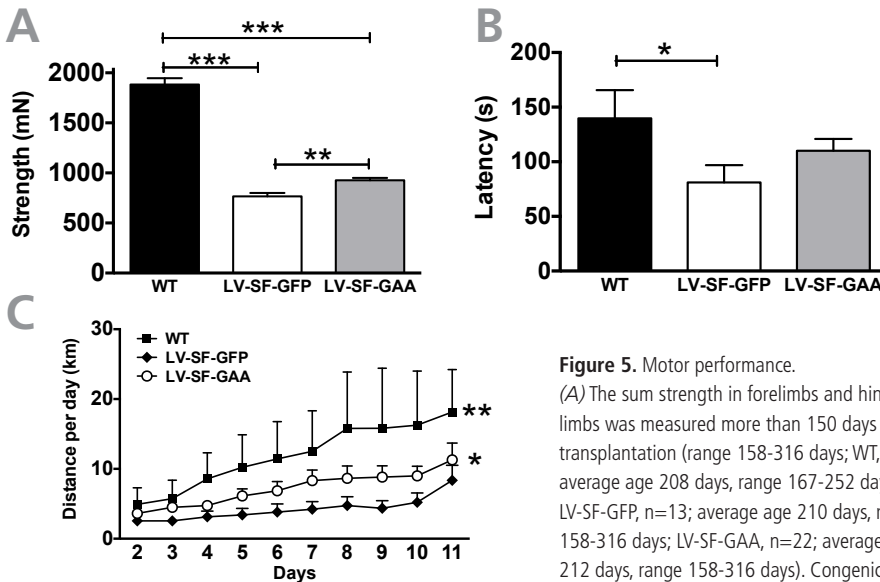


Figure 5. Motor performance.

(A) The sum strength in forelimbs and hind limbs was measured more than 150 days after transplantation (range 158-316 days; WT, $n=6$; average age 208 days, range 167-252 days; LV-SF-GFP, $n=13$; average age 210 days, range 158-316 days; LV-SF-GAA, $n=22$; average age 212 days, range 158-316 days). Congenic age matched WT animals are stronger than both LV-SF-GFP and LV-SF-GAA-transplanted $Gaa^{-/-}$

mice, and the LV-SF-GAA-transplanted mice are significantly stronger than LV-SF-GFP mice (** $P < 0.01$, *** $P < 0.001$).

(B) Rotarod performance. The latency (seconds on the rotarod) was determined in all treatment groups at the age of 10-months (WT, $n=6$; LV-SF-GFP, $n=10$; LV-SF-GAA, $n=14$). WT mice perform significantly better ($P < 0.05$) than LV-SF-GFP-transplanted animals. LV-SF-GAA-transplanted mice perform on average better than LV-SF-GFP-transplanted animals (not significant), but worse than WT animals (not significant).

(C) Running wheel performance. Running distances increased during the 11 days that they were measured (WT, $n=3$; LV-SF-GFP, $n=5$; LV-SF-GAA, $n=6$). WT mice run longer distances than both LV-SF-GFP (** $P < 0.01$) and LV-SF-GAA-transplanted mice, and LV-SF-GAA-transplanted mice perform better than LV-SF-GFP-transplanted animals ($P < 0.05$).

hematopoietic progenitor cells did not yield differences between WT and the other groups of mice as determined by enumeration of early (erythroid burst-forming unit) and late (erythroid colony-forming unit [CFU-E]) erythroid, granulocyte/ macrophage-CFU, and megakaryocyte-CFU progenitors (supplemental Table 5). By experimental design, the progeny of both the LV-SF-GFP and LV-SF-GAA-treated HSC represents a minority of the blood cells produced and, therefore, also of the progenitor cells. For this reason, this analysis does not fully exclude possible functional changes by over expression of GAA. However, the sustained and highly stable production of GAA under competitive repopulation conditions does not point to any impairment of transduced long-term repopulating cells.

DISCUSSION

Transplantation of genetically engineered HSCs after a clinically relevant subablative conditioning regimen leads to ectopic overexpression of GAA in PB cells with major clearances of glycogen in heart, diaphragm, stomach, uterus, lung, liver, and spleen. As a consequence, cardiac remodeling was reversed, respiratory function was improved, and skeletal muscle strength and motor performance were significantly ameliorated proportional to the reduction of glycogen storage. The presence of cells that overexpress GAA did not affect overall hematopoietic cell number and function. In line with the intrinsic tolerogenic nature of HSC transplantation and stability of enzyme levels up to the current observation time of 1.5 years, antibodies often observed in patients with Pompe's disease treated with ERT could not be detected after highly immunogenic challenging with human GAA.

Coexistence of a normal hematopoietic and immune system sustained from nontransduced stem cells and a minority of GAA-producing cells is an inherent strength of the experimental strategy and will facilitate potential clinical implementation. In the current experiments, leading to a high level of efficacy in adult mice, the contribution of transgene-expressing cells is on average roughly 35% of hematopoietic cells, which indicates that a low-dose cytoreductive conditioning regimen, such as the busulfan regimen applied in the successful clinical trial for adenosine deaminase-deficient severe combined immunodeficiency (SCID),³⁷ might well be sufficient in human patients. The minimum myelosuppressive conditioning regimen and stem cell numbers required for optimal efficacy need to be further established in a follow-up study along the lines described previously for α - and β -thalassemia,^{38,39} as is the minimum transgene copy number per cell.

At the time of treatment, the mice have glycogen deposition in heart, smooth muscle, and skeletal muscle, but overt pathologic manifestation occurs when mice age. Because patients severely affected with Pompe's disease die within the first year of life from cardiac and respiratory failure, early prevention or reversal of disease progression is essential for clinical efficacy. Because early intervention and high ERT dosing are essential requirements

to treat early-onset Pompe's disease successfully, transplantation of HSCs with high expression levels of the enzyme should have increased benefit if applied immediately after diagnosis.

Significant reductions of glycogen content and improved motor function have been obtained through systemic AAV vector administration.^{40,41} The response of skeletal muscles to genetically modified HSC transplantation is limited relative to that of heart and other organs. This problem is organ specific and also observed in enzyme replacement therapy. To further improve clearance of skeletal muscle fibers after gene-corrected HSC transplantation, enhanced secretion of chimeric GAA may benefit,⁴² as well as early intervention, ie, at the time of weaning or in neonates when glycogen deposition is still limited. Similar to ERT² and contrasting storage disorders characterized by severe neuronal impairment, genetically modified HSC transplantation does not influence glycogen deposition in brain tissue, which, however, does not elicit known clinical features in mice and humans and may therefore not be essential, but the option of clearance in brain tissue warrants further investigation.

Recently, it was reported that HSC expression of human GAA in combination with ERT could lead to partial correction and immune tolerance.¹⁸ Our results show that 30- to 50-fold levels in the hematopoietic system are well tolerated. These expression levels reduced glycogen content and improved cardiac remodeling significantly. In contrast, the low levels of GAA expression achieved in the study by Douillard-Guilloux et al¹⁸ required adjunctive ERT to reduce heart glycogen content. Conversely, in the latter report glycogen storage in the gastrocnemius muscle was significantly reduced without ERT, as opposed to a more limited, statistically insignificant reduction in glycogen content in the QF muscle examined in our study. However, the high GAA expression does significantly improve motor performance and, thus, resulted in long-term functional phenotype reduction.

During the near lifespan follow-up of the mice, neither numerical nor functional aberrancies in hematopoiesis were discerned. The risk of insertional mutagenesis is presently under active investigation. It is of note that the SIN-LV vectors used have a low genotoxicity profile relative to γ -retroviral vectors,⁴³ but clinical trials with the latter have also shown that insertional mutagenesis may have diverse outcomes, eg, development of clonal T-cell proliferation in the X-linked SCID trials^{44,45} and clonal dominance augmented by insertional activation in X-linked chronic granulomatous disease,⁴⁶ which may be partly related to the underlying disease. We do not per se advocate the use of the SF promoter for clinical application; however, aberrant clonal proliferation has not been observed in successful clinical γ -retroviral gene therapy trials for treatment of the enzyme deficiency adenosine deaminase-deficient SCID,^{37,47} both cases using viral promoters and one notably a similar promoter as in our Pompe study. More large-scale experiments are required to establish the safety profile of therapeutic LV vectors at disease-specific backgrounds. We prefer to replace the strong SF promoter by cellular promoters, such as the phosphoglycerate

kinase or elongation factor 1 α promoter, which are less likely to hit and activate cellular proto-oncogenes, as has been shown for *Evi1* by sensitive cell-based assays⁴⁸ and are currently developing sequence-optimized human *GAA* constructs to further improve protein expression per transgene copy. If this strategy works for the human *GAA* gene, the weaker cellular promoters should replace the SF promoter to achieve both therapeutic clinical efficacy and improved safety.

Allogeneic HSC transplantation has been explored for more than 2 decades as a treatment method for lysosomal storage diseases. Beneficial effects are achieved in Hurler disease (mucopolysaccharidosis I),¹¹ Krabbe disease (globoid cell leukodystrophy),¹² and MLD.⁴⁹ However, disease-related pathology progression by HSC transplantation is mostly partial and transplant-related morbidity and mortality remain significant hurdles. Overexpression of a corrective transgene in autologous HSCs would represent a major benefit, because it combines the advantages of a single intervention with the patient's own cells and that of ERT, as has been shown for MLD.¹⁶ In Pompe's disease, allogeneic HSCs would probably be ineffective because of the normally low levels of *GAA* activity in blood cells.

The present study convincingly shows that an efficient over-night transduction of HSCs results in genetically engineered progeny that overproduces *GAA*, which cross corrects affected cell types with prominent and sustained therapeutic efficacy, especially in the life-threatening cardiac and respiratory functional impairments as well as in complete clearance of glycogen in liver and spleen. Lifelong therapy by an endogenous transgene product is an obvious advantage over lifelong ERT and a prerequisite to clinical application. The results may contribute to further development of an efficacious *ex vivo* HSC-mediated gene therapeutic method that concomitantly induces immune tolerance to the transgene product.

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SUPPLEMENTARY DATA: FIGURES

High expression GAA lentiviral vector.

The LV-SF-GAA vector was titrated on *Gaa*^{-/-} mesenchymal stem cells (MSCs). MSCs were stained for acid α -glucosidase protein (Figure S1 A,B). The concentration of the LV viral vector preparation was 9.3×10^5 MSC transducing units (TU)/mL, which was about 5 times lower than the number of TU/ml generated with the LV-SF-GFP mock vector. For *ex vivo* experiments a LV vector preparation was used concentrated by ultracentrifugation to 2.3×10^8 TU/ml. Acid α -glucosidase activity was determined in transduced MSCs and corrected for the percentage of transduced cells. The activity of wild type (WT) MSCs was 15-fold higher than of *Gaa*^{-/-} LV-SF-GFP MSCs. The activity in LV-SF-GAA transduced *Gaa*^{-/-} MSC was 57-fold increased compared to the activity in WT MSCs showing that the LV vector was functional (data not shown).

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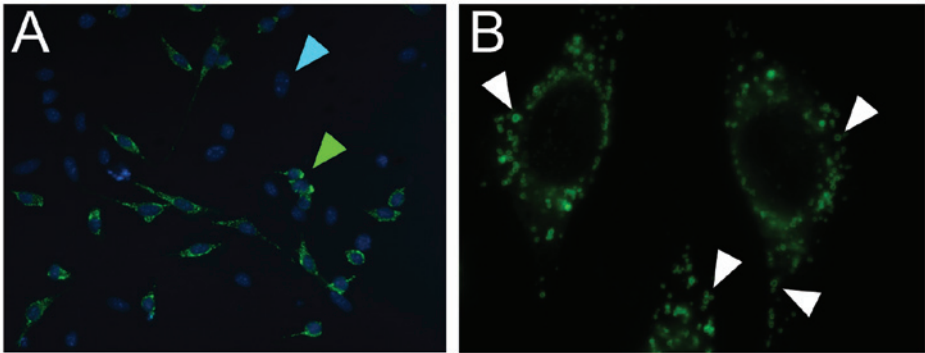


Figure S1: *In vitro* lentiviral vector expression of human acid α -glucosidase. Immunostaining of LV-SF-GAA transduced Pompe mouse MSCs. A) Nuclear DAPI staining (blue) was used to visualize the cells, immuno-staining (green) to visualize human GAA expression. Transduced GAA positive MSCs are clearly distinguishable from non-transduced MSCs. This staining method was used -next to Q-PCR- to titrate the LV-SF-GAA vector preparations (original magnification $\times 250$). B) This slide, only stained with anti-GAA antibodies, shows at higher magnification the typical lysosomal distribution of acid α -glucosidase (arrowheads) in transduced MSCs (original magnification $\times 1000$).

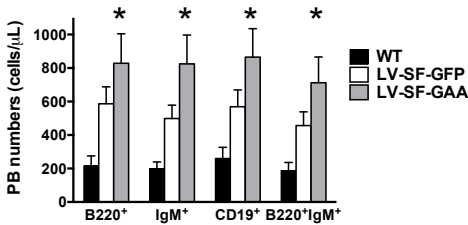


Figure S2: Peripheral blood B-cell numbers are elevated in LV-SF-GFP treated mice. Absolute peripheral blood (PB) B-cell markers B220⁺, IgM⁺, CD19⁺ and B220⁺IgM⁺ double positive cells were increased (* $p < 0.01$).

SUPPLEMENTARY DATA: TABLES

Table S1. Statistical analysis* of heart function tests.

<i>Comparison</i>	WT Versus LV-SF-GFP	WT Versus LV-SF-GAA	LV-SF-GAA Versus LV-SF-GFP
LV _{EDT} (mm)	<0.0001	0.0017	0.0027
LV _{EST} (mm)	<0.0001	0.0101	0.0296

*P-values for the echography tests determined by 2-way ANOVA for repeated measurements. WT mice were compared to the other groups at two time points. LV-SF-GFP was compared to LV-SF-GAA at three time points. LV_{EDT} left ventricle wall thickness at end diastole; LV_{EST} left ventricle wall thickness at end systole.

Table S2. Statistical analysis* of respiratory function tests.

<i>Comparison</i>	WT Versus LV-SF-GFP	WT Versus LV-SF-GAA	LV-SF-GAA Versus LV-SF-GFP
Peak inspiratory flow	<0.0001	0.0019	0.0169
Tidal volume	0.0001	0.0037	0.1056
Frequency	0.0001	0.0899	0.0088

*P-values for the respiratory function tests determined by 2-way ANOVA for repeated measurements.

Table S3. Statistical analysis* of distance covered in running wheels.

<i>Comparison</i>	WT Versus LV-SF-GFP	WT Versus LV-SF-GAA	LV-SF-GAA Versus LV-SF-GFP
Distance (km)	<0.0001	<0.0001	0.0153

*P-values for the running wheel experiments determined by 2-way ANOVA for repeated measurements.

Table S4. Complete blood counts*.

	WBC ('109/l)	RBC ('1012/l)	HB ('105)	HT ('105)	PLT ('109/l)	MCV	MHC	MCHC
WT (n=6)	1.97±0.60	8.36±2.37	7.77±1.89	40.5±9.0	780±194	49.7±6.6	0.95±0.13	19.1±0.9
LV-SF-GFP (n=7)	1.94±0.47	8.06±0.28	7.11±0.28	37.1±1.5	879±107	46.1±0.4	0.88±0.01	19.2±0.3
LV-SF-GAA (n=7)	2.21±0.90	7.92±0.33	7.14±0.28	36.6±1.6	999±142	46.1±0.9	0.90±0.03	19.5±0.6

*Blood counts determined in BM and spleen. WBC = white blood cell count, RBC = red blood cell count, HB=hemoglobin, HT= hematocrit, PLT= platelets, MCV= Mean Corpuscular Volume, MHC= Mean Corpuscular Hemoglobin and, MCHC= mean corpuscular hemoglobin concentration. All data is presented ± StDev.

Table S5. Progenitor colony assays*.

BM	Tnc ('106)	BFU-E ('105)	CFU-E ('105)	GM-CFU ('105)	CFU-Meg ('105)
WT (n=6)	16.8±5.01	56.7±10.1	306±212	185±47	38.2±15.5
LV-SF-GFP (n=7)	22.0±6.36	77.2±19.5	293±117	197±47	51.7±24.4
LV-SF-GAA (n=7)	19.4±2.73	68.2±20.7	251±107	184±41	40.0±14.8
Spleen	Tnc ('10 ⁶)	BFU-E ('10 ⁵)		GM-CFU ('10 ⁶)	CFU-Meg ('10 ⁶)
WT (n=5)	167±67	75.4±36.2		153±61	48.6±16.4
LV-SF-GFP (n=7)	201±35	73.6±28.6		159±35	63.9±9.9
LV-SF-GAA (n=7)	170±40	112.1±47.0		187±42	66.0±12.9

* Colony assays determined in BM and spleen. Tnc = total number of cells, Early (BFU-E), and late erythroid progenitors (CFU-E), granulocyte/macrophage (GM-CFU), and megakaryocyte (CFU-Meg) progenitors. All data is presented ± StDev.

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Stem cell based gene therapy

for Pompe's disease

acid alpha-glucosidase

Integrations

Hematopoietic stem cells

Muscle

Mouse

Single intervention

Lentivirus

Future

Storage

ERT

Transplantation

Costs

Glycogen

Immune tolerance

Transplantation

ABSTRACT

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Pompe's disease is an autosomal recessive lysosomal storage disease, characterized by progressive muscle weakness and death within the first year of life in its most severe form due to cardiac and respiratory failure. The disease is caused by mutations in the gene encoding acid alpha-glucosidase (GAA). GAA deficiency results in elevated levels of glycogen in virtually all tissues, most prominently in skeletal muscle. An integrating lentiviral vector with an optimized sequence of the human GAA gene driven by a strong viral promoter was used to transduce hematopoietic stem cells (HSCs), transplanted into 6-8 weeks old GAA deficient mice after mild conditioning. To determine the effect of the treatment, the mice were monitored monthly for hematopoietic parameters and enzyme activity, and sacrificed after 10-12 months. Life-long high levels of human alpha-glucosidase were achieved in leukocytes and in all affected tissues, including the skeletal muscle and brain of treated Pompe mice. Concomitant reduction in glycogen to normal levels was demonstrated in all tissues examined. Cardiac parameters were normalized, and the reduction of glycogen in skeletal muscle resulted in normal performance in voluntary running wheels. Glycogen reduction in the brain was likely attributable to correction of microglia cells descending from transduced HSCs. Presence of GAA was demonstrated in astrocytes that play a role in neuronal glucose and lactate metabolism. Insertional mutagenesis due to lentiviral integrations was not detected.

It is concluded that *ex vivo* hematopoietic stem cell mediated lentiviral vector gene therapy using a codon-optimized GAA expression construct can correct the Pompe phenotype in mice. The results warrant clinical implementation in the most severely affected infants with Pompe's disease.

INTRODUCTION

Pompe's disease (glycogenosis type II, acid maltase deficiency, OMIM 232300) is an autosomal recessive lysosomal storage disorder caused by deficiency of the lysosomal hydrolase acid alpha-glucosidase (GAA). The enzyme deficiency hampers the breakdown of glycogen to glucose and leads to systemic accumulation of glycogen inside the lysosomes¹⁻⁴. The disease manifests with a broad clinical spectrum, ranging from the most severe and rapidly progressive classic-infantile form to less progressive forms with usually later onset⁵. Classic-infantile Pompe's disease is characterized by profound skeletal muscle weakness, respiratory distress and cardiomyopathy, where death occurs within the first year of life if untreated⁶. The lysosomal glycogen storage is generalized and has, for instance, also been observed in articular cartilage^{7, 8}, smooth muscle cells of blood vessels and intestinal tract, and in neurons of the peripheral (PNS) and central CNS) nervous system⁹. By absence of cardiomyopathy in childhood and adult forms of Pompe's disease progressive skeletal muscle weakness becomes the main clinical feature and poor pulmonary function the most life-threatening event³.

Enzyme replacement with recombinant human GAA (rhGAA, Myozyme[®]) provides a therapy for patients with Pompe's disease. They receive weekly or bi-weekly intravenous infusions of the enzyme, which is internalized by the cells via the mannose 6-phosphate receptor. Despite an overall better survival and improved quality of life, enzyme replacement therapy (ERT) is not considered the ultimate solution since residual disease remains in most cases and neutralizing immune responses can occur¹⁰⁻¹⁵. Moreover, ERT cannot cure CNS pathology, would this occur, as the enzyme (rhGAA) is believed to not cross the blood-brain barrier^{16,17}.

Alternative or adjuvant approaches to treat Pompe's disease include *in vivo* gene therapy by adenoviral vectors and adeno-associated virus vectors (AAV),^{18,19} suppression of autophagy²⁰ or suppression of glycogen biosynthesis²¹, and the use of chaperones in addition to ERT for proper folding and stability of the therapeutic protein (rhGAA)²². However, long-term efficacy has not been demonstrated for any of these approaches.

Ex vivo hematopoietic stem cell (HSC) based gene therapy could possibly overcome this problem as it has been shown to provide a continuous supply of GAA inherent to the perpetual production of blood cells. Previously, we have shown that *ex vivo* lentiviral gene therapy using HSCs ameliorates the phenotype of Pompe mice, although the enzyme levels obtained were not sufficiently high to achieve full correction of the glycogen storage in skeletal muscle²³, a similar problem is encountered with ERT^{24,25}. We have also shown the induction of robust immune tolerance to ERT by *ex vivo* HSC based gene therapy, which is attributable to the intrinsic tolerogenic nature of HSC transplantation. Aiming at complete correction of the Pompe phenotype in mice by a minority of gene-modified HSCs, we examined codon-optimization²⁶ of the therapeutic transgene to achieve very high enzyme levels.

METHODS

Animals

Gaa^{-/-} mice (FVB/N background) were generated as previously described²⁷. Age-matched control female FVB/N mice were obtained from Charles River Nederland (Maastricht, the Netherlands). All procedures were approved by the animal ethical committee of the Erasmus University Medical Center, Rotterdam in accordance with legislation in the Netherlands.

Construction of vector plasmids

The human *GAA* native construct pRRL.PPT.SFFV.GAA.WPRE*.SIN (*GAA*) was generated as described previously^{23,28}. The OptimumGene™ algorithm (Genscript, Piscataway, NJ, USA) was used to optimize the human *GAA* open reading frame including the consensus Kozak sequence for improved expression and an extra TGA stop codon was added (*GAAco*). *GAAco* was cloned into the pRRL.PPT.SFFV.GFP.bPRE4*.SIN replacing *GFP*, and creating pRRL.PPT.SFFV.*GAAco*.bPRE4*.SIN (*GAAco*).

Transduction and transplantation of murine HSC

Bone marrow was harvested from the femora and tibiae of 8-12 weeks old male *Gaa*^{-/-} mice (KO). Lineage negative (Lin⁻) cells were purified using the mouse hematopoietic stem cell isolation Kit (BD Biosciences) and transduced at a multiplicity of infection of 20 with the *GAAco* vector and a multiplicity of infection of 2 for the GFP vector, the latter to serve as for mock therapy (these mice are marked as KO). Cells were cultured overnight in Dulbecco's medium, supplemented with the growth factors: murine-stem cell factor (100 ng/mL), human FMS-like tyrosine kinase 3 murine ligand (50 ng/mL), murine thrombopoietin (10 ng/mL). Transduced cells (5x10⁵ cells) were transplanted via the tail vein of 6-8 week-old *Gaa*^{-/-} female recipients after a sublethal conditioning regime of 6 Gy.

Tissue preparation and histology

At 10-12 months after transplantation, the mice were fasted overnight and sacrificed by transcardial perfusion with saline after administration of ketamine/sedator (Eurovet) mix. Tissues were collected and homogenized by TissueLyserII (Qiagen), 2 times for 2.5 min at 30 Hz. Histological assessments were performed as described earlier²³ with the exception of cartilage. The tibialis bones were collected, undecalcified fixed and embedded in methylmethacrylate (MMA). PAS (Periodic Acid Schiff for glycogen) staining was performed as previously described²⁹. PAS positive chondrocytes were counted as percentages of the total number of chondrocytes that were scored.

Immunofluorescence staining

For localization of GAA in the brain, transplanted mice were fasted overnight and sacrificed by transcardial perfusion with 4% formaldehyde (FA) in phosphate-buffered saline (PBS). Whole brain was carefully dissected out, overnight fixed in 4% FA, before frozen in O.C.T. Tissue Tek (Sakura Fintek Europe) and sectioned at 6 μm . Sections were permeabilized with acetone and methanol (1/4 vol/vol) and then immersed in 10% fetal calf serum (FCS) in PBS at room temperature. Brain sections were stained for the presence of GAA using a 1:100 dilution of rabbit anti-human recombinant GAA serum. Astrocytes were identified using anti-gial fibrillary acidic protein (GFAP; Sigma-Aldrich; 1:500) and microglia cells using F4/80 (AbD Serotec; 1:200) overnight at 4°C. Sections were washed with PBS and incubated with secondary antibodies AlexaFluor488/555/647, (Invitrogen; 1:500) at room temperature. Hoechst (Sigma Aldrich) was used for nuclear staining. Tissue sections were examined using fluorescence microscopy (Leica DMRXA) equipped with the Leica DFC 350FX camera, or imaged with a Leica SP5 CLSM equipped with Argon/HeNe lasers using a 63x Planapochromat oil immersion objective. The scanned stacks were deconvolved and visualized with Huygens Professional 4.2 (Scientific Volume Imaging, Hilversum, The Netherlands).

GAA activity and glycogen assays

GAA activity and glycogen content were determined as described previously^{30,31} and corrected for protein content (BCA kit, Pierce).

Quantitative polymerase chain reaction of LV integrations

Spleen cell DNA was extracted with the NucleoSpin Tissue kit (Bioké) to determine the vector copy per cell by quantitative PCR (q-PCR) with the following primer set: HIV- ΔU3 -forward primer, 5'-CTGGAAGGGCTAATTCCTC-3', and HIV-reverse primer, 5'-GGTTCCCTTC-GCTTCAG-3' on the ABI PRISM 7900 HT sequence detection system (Applied Biosystems). To determine the chimerism, Y-chromosome specific *Sry* locus primers forward, 5'-CATCGGAGGGCTAAAGTGTCAC-3', and reverse 5'-TGGCATGTGGGTTCTGTCC-3' were used. Samples were normalized to mouse *Gapdh* (forward primer, 5'-CATCACTGC-CACCCAGAAGAC-3', and reverse primer, 5'-TGACCTTGCCCACAGCCTTG-3'). Samples were analyzed with SDS2.2.2 software (Applied Biosystems).

Echocardiography

Ten to twelve months after transplantation, mice were weighed, anesthetized with isoflurane, and intubated as previously described³². Animals were placed on a heating pad to maintain body temperature at 37°C while being artificially ventilated with a mixture of O₂ and N₂O (1:2 (vol/vol)) to which isoflurane (2.5% (vol/vol)) was added at a rate of 90 strokes/min using a rodent ventilator (SAR-830/P, CWE; Ardmore, PA) at an inspiratory

pressure of 18 cm H₂O. To dehair the chest, Veet hair removal cream (Reckitt Benckiser; Parsippany, NJ) was used. Transthoracic echocardiograms of the left ventricle (LV) were obtained with an Aloka SSD 4000 echo device (Aloka; Tokyo, Japan) using a 13-MHz probe. Images of the short and long axis were obtained in two-dimensional and M-mode settings, used to measure the left ventricular lumen diameter and LV wall thickness at end diastole and end systole by SigmaScan Pro 5 Image Analysis software.

Muscle performance tests

To determine skeletal muscle function, mice were tested at 10-12 months after transplantation on an accelerating rotarod (from 4 to 40 rpm in 5 minutes; Panlab/Harvard Apparatus Rota Rod). Mice were given 2 training sessions to adjust to the apparatus, followed by 3 runs with an interval of 5 minutes and the latencies were then averaged.

Voluntary wheel running was used to measure activity performance at 10 months after transplantation for 28 days. Mice were individually housed in type III polyester cages, equipped with a steel running wheel (diameter 11.8 cm) connected to a sensor system to detect revolutions of the wheel³³. Voluntary wheel running (number of revolutions per 2 min) was continuously recorded using the ERS program (University of Groningen, the Netherlands). The distance the mice ran daily during 28 days was calculated by multiplying the number of revolutions with the circumference of the wheels.

LAM-PCR

High-resolution insertion-site analysis by linear amplification-mediated PCR (LAM-PCR)³⁴ was performed on bone marrow or thymus DNA from thirteen *Gaa*^{-/-} mice transduced with SF.GFP or SF.GAAco LV vectors. Restriction enzyme Tsp509I was used with the lentiviral (HIV) primer set, and PCR products were run on high resolution polyacrylamide (Spreadex) gel. Sequencing reads were analyzed using MAVRIC. Sequences were aligned to the murine genome via BLAST (v 37) and nearby genes identified using Ensembl version 59.

Statistical analysis

Statistical analysis was performed with Graph Pad Prism. Significance of differences was determined by one-way analysis of variance or Mann-Whitney U test for comparing two groups. For repeated measurements two-way ANOVA was used to determine differences between groups. Significance of a difference was assumed when the p-value <0.05. Error bars represent SEM.

RESULTS

Recoding GAA improves enzyme activity

Male Lin⁻ bone marrow cells transduced with either the codon-optimized GAA expression construct (GT) or a construct expressing GFP (used as a negative control and throughout the manuscript made as KO), both driven by the spleen focus forming virus promoter (SF) were compared to a construct containing the native GAA sequence with regard to GAA expression²⁹. At 10-12 months after transplantation of the gene-modified Lin⁻ cells, supranormal GAA enzyme levels were measured in leukocytes and spleen cells of gene therapy treated animals (Supplemental data Figure 1). The GAA activity per vector copy number (VCN) in spleen was increased with the recoded GAAco expression construct compared to the GAA construct (Supplemental Figure 2). Transduced male donor cells transplanted into sublethally irradiated female recipients resulted in a chimerism in the spleen similar in both the GAA and the GAAco transplanted animals (on average $24.1 \pm 5.0\%$).

Efficient delivery of GAAco to affected tissues

Cross-correction of affected tissues was investigated by determining the GAA activity in different tissues. The GAA activity increased in tissues e.g. liver, kidney, lung and stomach up to 18-fold wild-type levels (Figure 1A).

To evaluate the therapeutic effect of supranormal enzyme levels, the quantity of glycogen within each tissue was measured (Figure 1B). As expected, the very high enzyme activity resulted in almost complete reduction of glycogen within the evaluated tissues. In mice that received the mock vector (KO), massive glycogen deposition was observed whereas wild-type levels were obtained in lung, liver, spleen and kidney after GAAco gene-modified HSC transplantation.

Efficient delivery of GAAco to affected skeletal muscles

Using the codon-optimized construct all gene-therapy treated animals showed sustained GAA activity levels at 10-12 months after transplantation. Notably, supranormal enzyme levels were also obtained in skeletal muscles (Figure 2A) *i.e.*, tongue, masseter, quadriceps femoris, gastrocnemius, soleus, extensor digitorum longus (EDL) and tibialis anterior (TA). The fold increase of enzyme activity in muscle was similar as the increase visceral organs, and consistent with substantial reduction of glycogen storage (Figure 2B).

Delivery of GAAco restores skeletal muscle function

Treated, untreated and wild-type mice were subjected to rotarod and voluntary runningwheel tests to relate locomotor function to the profoundly reduced glycogen levels observed. Measuring the latency on an accelerating rotarod at 10 months after transplantation showed correction of the skeletal muscle function of the GAAco treated mice

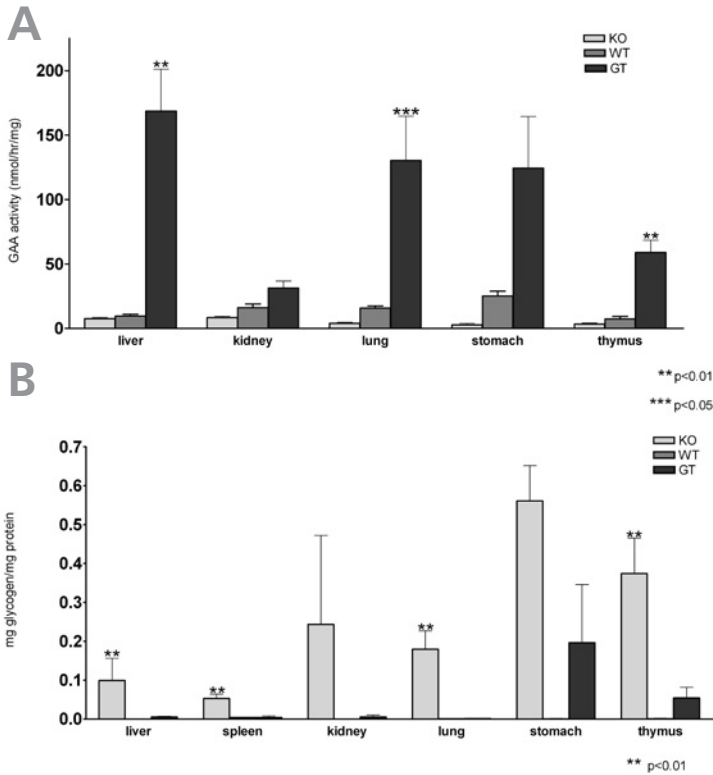


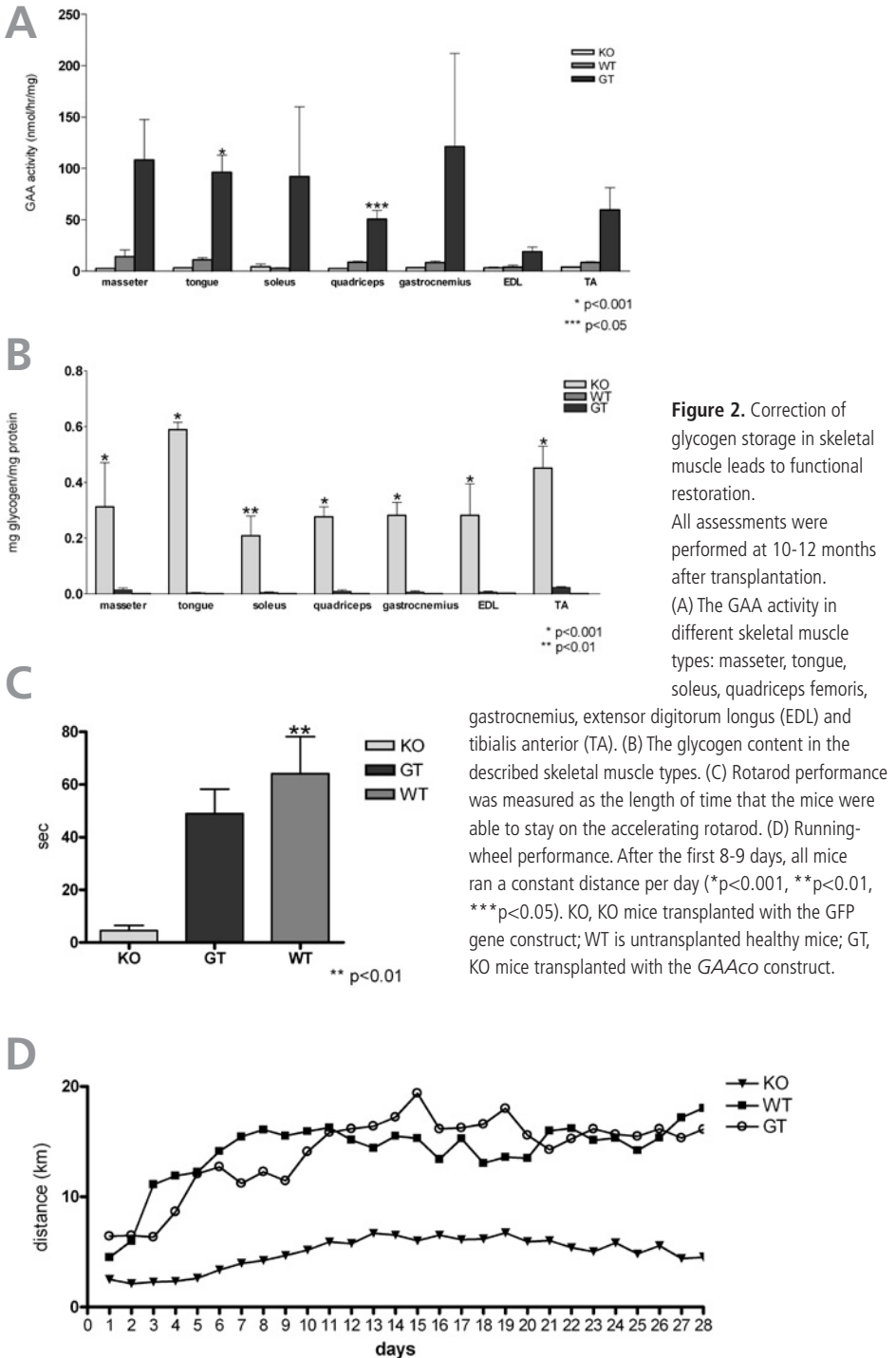
Figure 1. Effect of treatment with SF.GAAco modified HSCs.

Gaa^{-/-} mice were transplanted with 5×10^5 Lin⁻ cells transduced with the therapeutic vector SF.GAAco at a MOI of 20 (GT) or GFP as negative control (KO). Ten months after transplantation, we measured both the GAA activity (A) as well as the glycogen content (B) in various tissue types. Age-matched wild type animals (WT) were used as positive controls (** $p < 0.01$, *** $p < 0.05$).

to a level approaching wild-type (Figure 2C) whereas the untreated mice were severely impaired. Similarly, voluntary runningwheel activity, monitored for 28 days, displayed no difference between the wild-type and GAAco treated animals, whereas the mock treated mice (KO) mice showed a slight improvement during the first 2 weeks of training, but were significantly less active than the wild-type mice (Figure 2D).

Delivery of GAAco corrects GAA deficiency, glycogen storage, and cardiac dimension and rhythm

To evaluate the effect of *ex vivo* gene therapy using the GAAco gene construct on heart, we measured the GAA activity, the glycogen content, and the cardiac rhythm and dimension by trans-cardiac echography (Figure 3C) at 10 months after transplantation. Treatment with the GAAco construct was sufficiently effective to fully correct the GAA deficiency (Figure 3A) and to significantly reduce the glycogen storage (Figure 3B). Echography revealed a marked left ventricle hypertrophy in the KO mice compared to the wild type mice and the



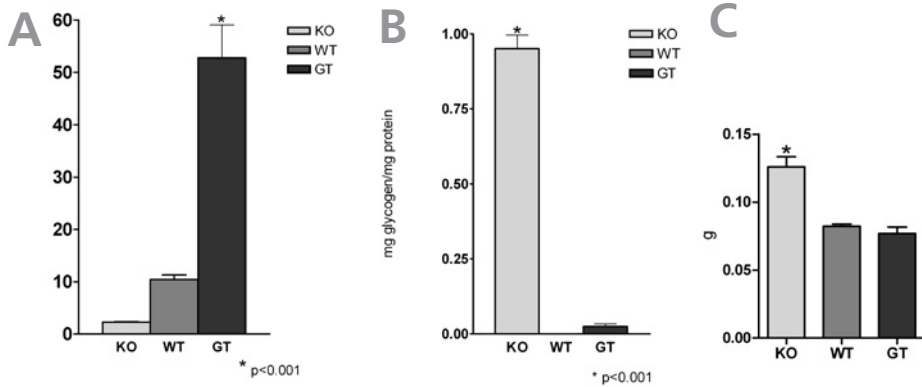


Figure 3. Delivery of GAAco corrects GAA deficiency, glycogen storage, and cardiac dimension.

All assessments were performed at 10-12 months after transplantation. (A) Correction of GAA deficiency in mice transplanted with the GAAco construct (GT) compared to mock treated KO mice. (B) Significant reduction of the glycogen content of the heart. The cardiac hypertrophy is corrected as illustrated by significant reduction of the total cardiac wet-weight in grams (C). (* $p < 0.001$, ** $p < 0.01$, *** $p < 0.05$).

mice transplanted with the GAAco construct; the cardiac remodeling due to transplantation was significant. Figure 3C illustrates the remodeling in terms of reduction of the total cardiac wet-weight in grams. The LV wall thickness and the LV lumen diameter normalized completely (data not shown). Other evidence of cardiac remodeling was the correction of the cardiac rhythm from 406.0 ± 44.8 bpm (KO mock treated mice) to 486.1 ± 49.0 bpm in the KO mice transplanted with the GAAco construct (GT); Wt, 467.1 ± 106.8 bpm).

High GAA expression reduces glycogen storage in articular cartilage

Sections of the tibiofemoral joint cartilage (Figure 4) were PAS-stained to demonstrate glycogen storage in the cartilage. Profound glycogen storage was found in the articular chondrocytes of mock treated KO mice, while no apparent glycogen deposition was detectable in WT mice. Mice treated with the GAAco construct had a visible reduction of glycogen storage in the articular chondrocytes at 10-12 months after transplantation: PAS staining showed 45% positive cells in GAAco treated animals compared to 93.75% glycogen positive cells in KO mice (The PAS staining intensity of WT chondrocytes was set at 0%). In addition to increased PAS staining intensity we noted chondrocyte hypertrophy in the articular cartilage of KO mice, while the cartilage of GAAco mice appeared morphologically normal compared to age-matched WT mice.

Correction of GAA deficiency and glycogen storage in the brain

Transplantation of 6-8 weeks old KO mice using the GAAco construct led to a significant increase of GAA activity to a level of approximately 50% of that in WT mice (Figure 5A) and to a significant reduction of the glycogen content (Figure 5B) of whole brain tissue when analyzed 10-12 months after transplantation. PAS staining of glycol methacrylate

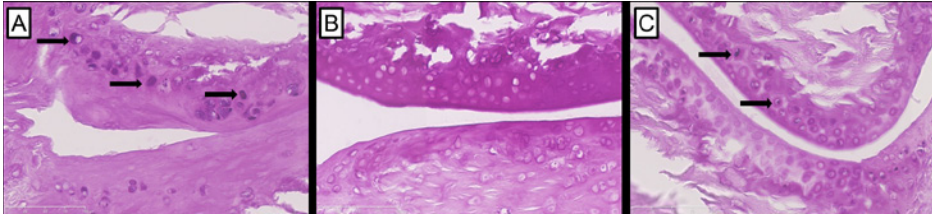
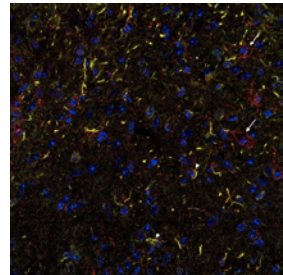
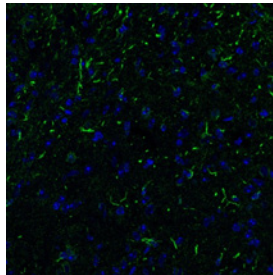
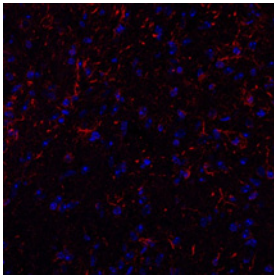
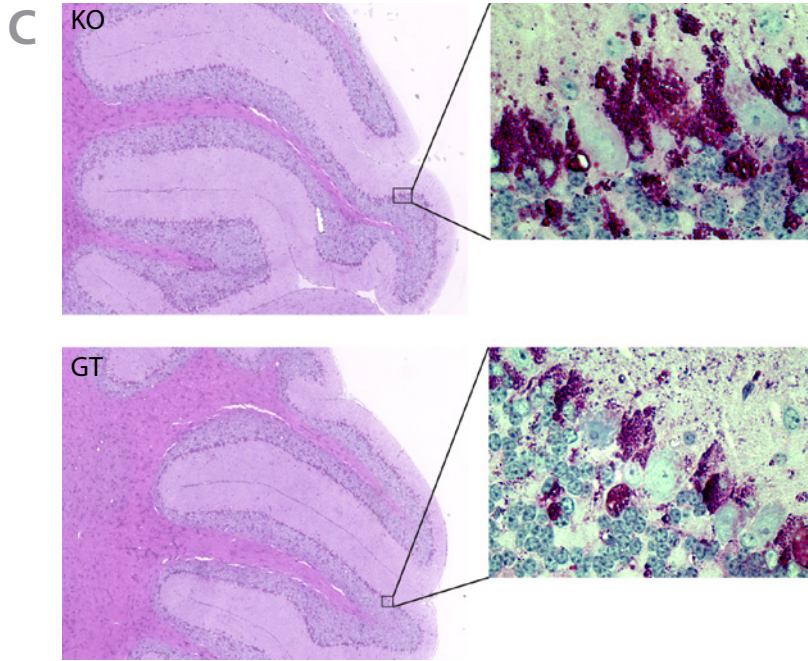
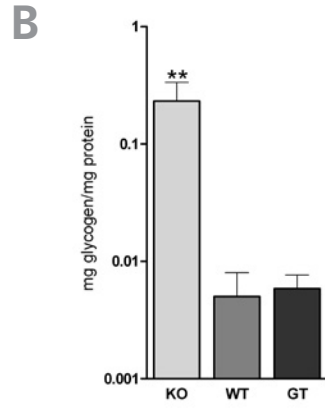
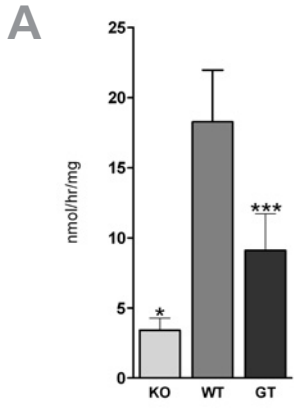


Figure 4. Reduction of glycogen storage and chondrocyte hypertrophy in articular cartilage by ex vivo gene therapy. This figure shows articular cartilage of the knee joint stained with PAS revealing the glycogen storage (arrows). The PAS staining is quite intense in cartilage of KO mice (A) and much reduced in cartilage of mice transplanted with the GAAco construct (C). Chondrocytes in WT cartilage hardly stain (B).

(GMA)-embedded brain slices confirmed these findings (Figure 5C). The strongest staining was observed in areas directly surrounding the Purkinje cells in the cerebellum of mock transplanted mice (Figure 5C, upper panel). The staining intensity was much less in mice transplanted with the GAAco construct (Figure 5C, lower panel). The area surrounding the Purkinje cells is mainly occupied by astrocytes (Supplemental Figure 3). Immunofluorescence shows a distinct localization of GAA in astrocytes (100% GAA positive) and microglia cells (50% GAA positive) (Figures 5D and E).

Integration site analysis and safety profiling

Bone marrow DNA from ten *Gaa*^{-/-} mice transduced with GFP or GAAco and thymic DNA transduced with GAAco were used as templates in LAM-PCR reactions prior to high-throughput sequencing. A total of 466 unique integration sites were mapped to the murine genome: 435 from mice treated with GFP and 31 from mice treated with GAAco. Integrations were annotated to the gene with the nearest transcription start site. Using this approach, the genes nearest each integration were sorted into ten bins based on their expression levels in lineage negative Sca-1⁺ C-kit⁺ (LSK) cells, with the higher expression bins being slightly favoured (Supplemental Figure 4). Genes were screened against a list of 528 oncogenes compiled by the RTCGD database. Thirteen oncogenes were found in the annotation of the integrations in GAAco treated mice (3.1% of the sample pool), with one oncogene, *Zfp521*, found twice. This is slightly less than the expected frequency of integrations near oncogenes found in *in vitro* studies of wild type murine lineage negative cells transduced with GFP (48 of 1285 ie 3.7%, data not shown), and very close to the frequency expected by random chance. Eight genes were annotated three or more times, and so are considered common integration sites (CIS). Most of the CIS integrations were intronic, none of the CIS genes were marked as oncogenes, and none of the CIS genes were present in the two highest LSK expression bins. Hits were widely distributed across the genome (Supplemental Figure 4B), with no integration “hot spots” in evidence.



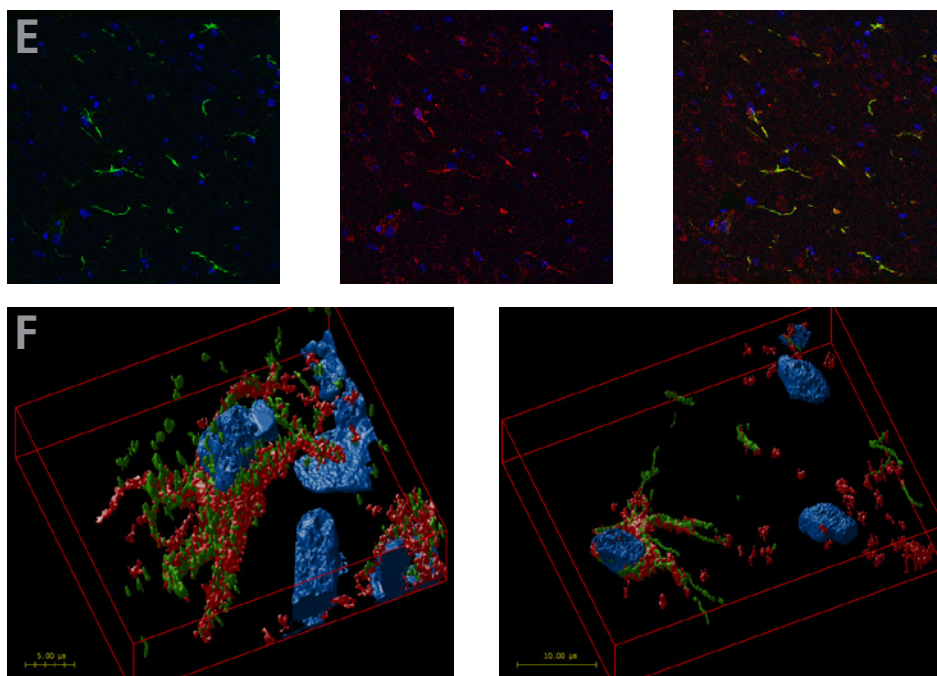


Figure 5. Effect of treatment with GAAco modified HSCs in brain.

The GAA enzyme activity (A) as well as the glycogen content (B) was measured in the brain of wild type, KO and GAAco treated animals at 12 months post transplantation. (C) H&E (left) and PAS (right) staining of the cerebellum. PAS staining shows a clear reduction of the glycogen (dark purple) in the brain of GAAco treated mice, bottom figure (magnification 1000x), compared to KO mice (top figure). H&E staining (magnification 63x) provides an overview of the brain. (D) Microglia staining (F4/80), anti-GAA staining and Hoechst staining: around 50% of the microglia cells stains positive for GAA (arrowheads). The arrows point to microglia cells negative for GAA. The astrocytes (GFAP staining) all show GAA content (E) Microglia staining (red, left panel) and anti-GAA staining (green, left panel) are in deconvolved mode (F) shown in the panel on the left. On the right, red represents GAA staining and green the astrocyte staining. In both deconvolved pictures, blue represents the nuclear Hoechst staining. (* $p < 0.001$, ** $p < 0.01$, *** $p < 0.05$).

DISCUSSION

As opposed to enzyme replacement therapy aiming to keep the lysosomal glycogen accumulation below the pathologic threshold by weekly or biweekly enzyme infusions, gene therapy approaches aim to introduce a long lasting supply of curative enzyme by one time genetic reprogramming of the patients cells through supplementing the missing genetic information, either *in vivo* or *ex vivo*³⁵. The current attempts at gene therapy in Pompe's disease are aimed at complete and sustained resolution of the glycogen storage within the skeletal muscles, which is also a current challenge for enzyme replacement therapy³⁵ that largely corrects cardiac function, but only partially corrects the respiratory problems and the strength of skeletal muscles. Our previous attempts to correct the Pompe's disease phenotype by *ex vivo* lentiviral mediated gene transfer via hematopoietic stem cells

encountered the same problem. A possible obstacle to efficiently delivering the enzyme to skeletal muscle can be the low density of the mannose 6-phosphate receptor in skeletal muscle tissue compared, for instance, to the high density of the mannose receptor on macrophages in relation to the treatment of Gaucher disease^{36,37}. However, such comparisons are not totally meaningful the target tissue itself and its accessibility pose unique challenges to the treatment of each lysosomal storage disorder per se. In this study, we have investigated the possibility of enhancing the effect of HSC targeted gene therapy by using a codon-optimized *GAA* construct (*GAAco*) to obtain high enzyme production. The construct consists of a strong viral promoter in a self-inactivating (SIN) third generation HIV-1 based modified lentiviral vector^{28,38,39}. This approach to obtain maximal ectopic gene expression resulted in a 40-fold increase of the *GAA* activity per integration, as compared with the native construct, and has met the aim of fully correcting the glycogen accumulation in skeletal muscle. The reduction of glycogen storage in other organs as well, surprisingly including the brain, was an unexpected and surprising finding.

Resolution of glycogen storage was demonstrated in the quadriceps femoris, the gastrocnemius, the extensor digitorum longus, the tibialis anterior, the soleus, the masseter and the tongue as well as in the heart, the thymus, the lungs, the diaphragm, the liver, the spleen, the stomach, the kidney and the uterus. The voluntary running wheel and rotarod tests confirmed that the restoration of enzyme activity not only resulted in lysosomal glycogen clearance but also in complete functional recovery of locomotor function. Interestingly, articular cartilage of mice transplanted with the *GAAco* construct displayed a major reduction in intracellular glycogen storage despite the fact that chondrocytes are particularly difficult to target due to the composition and the molecular density of the surrounding matrix⁴⁰. The amount of glycogen naturally decreases with the cellular differentiation state of bone⁴¹ and bone marrow⁴². In contrast, it increases during chondrogenic differentiation and glycogen is usually absent in superficial articular chondrocytes⁷ but rather characteristically present in deep-zone hypertrophic cells⁴³. Glycogen has an established function in normal chondrocyte physiology⁴⁴. Not surprisingly, chronic joint conditions and cartilage ulceration are reported in other glycogen storage disorders (like e.g. type I, van Gierke's disease)⁴⁵, but articular hypertrophy and inflammation has to the best of our knowledge not been reported in Pompe's disease.

Astrocytes have complex functions including development, maintenance and modulation of the blood-brain-barrier, control of energy supply in particular via glucose metabolism, and specific neuronal capacities such as long-term memory. The control of glycogen metabolism in the brain is even more complex than in muscle and liver⁴⁶. The glycogen store in astrocytes is modulated by the glucose concentration of the neuronal cells and by several other factors such as neurotransmitters. Glycogen is stored in the form of glycogen granules throughout the cytoplasm of the astrocytes, but most so in the peripheral processes that surround the neuronal elements. In this way, astrocytes support the neurons by

providing energy to neurons when their demand is high⁴⁷. Within the cerebellum, specialized astrocytes, Bergmann glia (also known as Golgi epithelial cells) form a specialized layer of supporting cells intermingled with the signal integrating Purkinje cells and form complex interactions with these cells⁴⁸⁻⁵⁰. Our method of detection does not discriminate between the cytoplasmic, i.e. the physiologic glycogen content, and the pathologic lysosomal content of these cells. However it is unquestionable that the total glycogen content of the astrocytes is significantly reduced by the *ex vivo* HSC mediated gene therapy using the GAAco construct to the extent that the glycogen content of whole brain tissue returns to normal levels.

Since starvation does not reduce the cytoplasmic glycogen reserves in the brain⁴⁶ as it does for instance in the liver, residual glycogen is observed also in the treated animals. Astrocytes have mannose 6-phosphate receptors on their surface, by which the GAA can be internalized⁴⁸. Intravenously administered recombinant human GAA is assumed not to pass the blood-brain barrier. The gene therapy approach described here resulted in vastly increased levels of GAA activity and decreased glycogen content of the brain, as evidenced by biochemical assays and PAS staining. The mechanism by which this occurs can be explained in either of two ways: i) GAA produced by genetically corrected white blood cells have crossed the blood-brain-barrier or ii) transduced myeloid cells have accessed the brain and deposited the enzyme locally⁵¹.

We have explored in this study the potential advantage of using a codon-optimized GAA expression construct over an unmodified GAA construct in a gene therapeutic approach to the treatment of Pompe's disease. *Ex vivo* HSC targeted and mediated gene therapy with a GAA codon-optimized construct clearly led to better outcome than obtained with the unmodified GAA construct. It resulted in normalization of the tissue glycogen content and functional correction of the target tissues. When our experiments in mice can be translated to humans it seems that a single gene-therapeutic intervention via the patients' own HSC can be sufficient to warrant effective and sustained delivery of GAA to the affected tissues, including the difficult to correct tissues like skeletal muscle and brain. As other advantage over ERT, *ex vivo* gene therapy by HSCT does not induce an immune response to the recombinant GAA thanks to the intrinsic nature of the procedure and the cell type used⁵². Although integrating lentiviral vectors are considered as rather safe since they do not have a preference for transcriptional start sites as compared to retroviral vectors, the use of these vectors still bears the risk of insertional mutagenesis⁵³. The lentiviral vector based GAA expression construct that we used for demonstrating the application of HSC mediated gene therapy in mice with Pompe's disease did not show increased prevalence for integration near proto-oncogenes and/or tumor-suppressor genes.

In conclusion, we have demonstrated that recombinant human GAA can be effectively delivered to murine tissues by HSC mediated lentiviral vector based gene therapy approach. To date, this is the only report that shows full clearance of the accumulated glycogen and

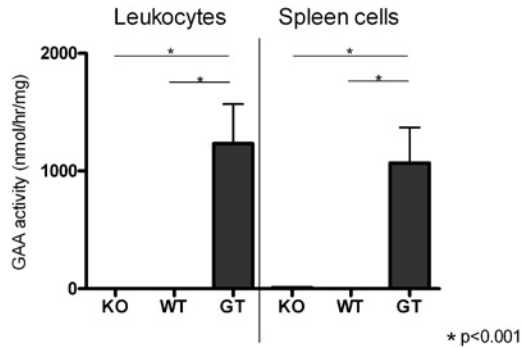
further prevention of storage of glycogen in skeletal muscle by a single intervention. Glycogen storage in the brain was partly prevented. These findings are a reason to further explore the application of HSC mediated lentiviral vector based gene therapy in Pompe's disease and related lysosomal storage disorders including diseases with CNS involvement.

ACKNOWLEDGEMENTS

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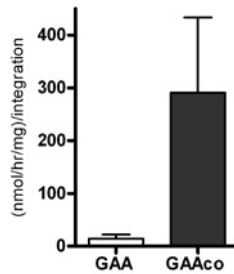
The authors thank Elza van Deel for performing the echocardiographies, Ton de Jong and Nicole Kops for their assistance with histochemistry. This work was supported by the European Commission's 5th, 6th, and 7th Framework Programs (contracts QLK3-CT-2001-00 427-INHERINET, LSHB-CT-2004-005242-CONSERT and grant agreement no. 222878-PERSIST), by The Netherlands Organization for Health Research ZonMW (program grant 434-00-010), and The Netherlands Organization for Scientific Research NWO (project 021.002.129). E.H.J. was supported by a NWO ZonMW-Veni Award (40-00806-98-06082) and an ERAB: The European Foundation for Alcohol Research project grant (EA 07 14).

SUPPLEMENTAL DATA



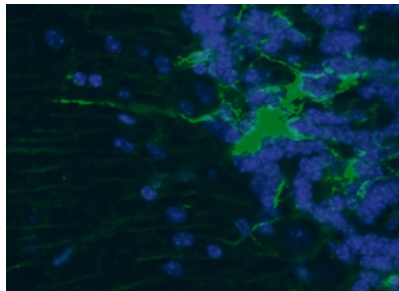
Supplemental Figure 1. Effect of treatment with GAAco modified HSCs in leukocytes and spleen cells.

Gaa^{-/-} mice were transplanted with 5×10^5 Lin⁻ cells transduced with the therapeutic SF.GAAco vector at a MOI of 20. Age-matched WT mice and mock treated mice (KO) were taken as positive and negative controls, respectively. WT mice have endogenous GAA expression (leukocytes: 4.1 nmol/hr.mg on average, and spleen cells 8.15 nmol/hr.mg on average). Enzyme expression was measured at 10 months after transplantation in peripheral leukocytes and spleen cells (*p<0.001).



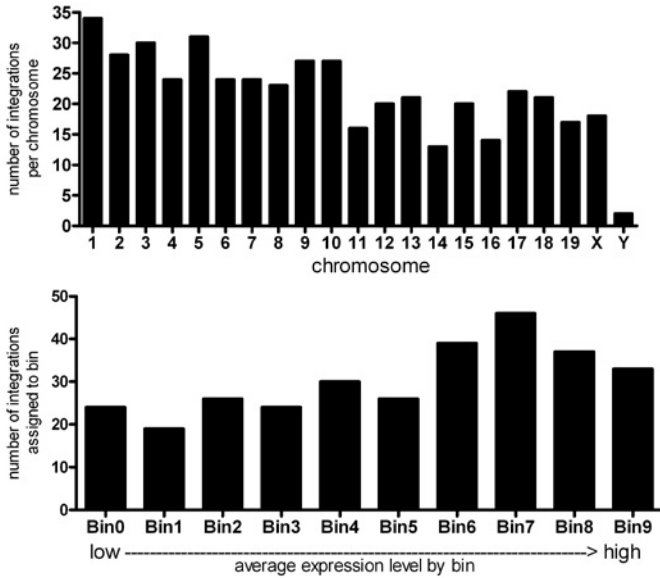
Supplemental Figure 2. GAA activity per integration.

The number of viral integrations per male donor cell was determined by q-PCR. When evaluating the GAA activity per viral integration, the GAAco construct generates an overall much higher activity per integration than the native GAA construct.



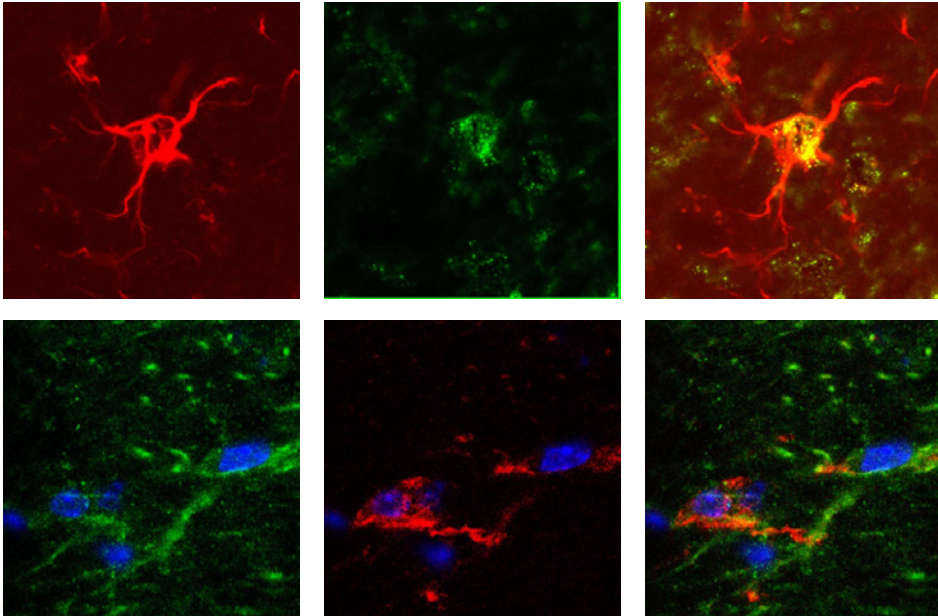
Supplemental Figure 3. Localization of neurons and astrocytes in the cerebellum.

To identify the strongly PAS positive cells surrounding the Purkinje cells (Figure 5C), we stained sections of the cerebellum with an astrocyte specific antibody (glial fibrillary acidic protein, GFAP; green). Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole; blue). Magnification 63x.



Supplemental Figure 4. Integration analysis.

Supplemental Figure 4A shows the number of lentiviral integrations for each chromosome. Figure 4B represents the number integrations per assigned bin.



Supplemental Figure 5. Immunofluorescence.

Figure A shows a high magnification (100x) of a GAA (green) positive astrocyte (red) and a merged picture in the right panel. GAA positive (red) microglia (green) staining, merged in the right panel (100x magnification). Hoechst (blue) was used as nuclear staining.

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Stem cell based gene therapy

for Pompe's disease

acid alpha-glucosidase

Integrations

Hematopoietic stem cells

Muscle

Mouse

Single intervention

Lentivirus

Future

Storage

ERT

Transplantation

Costs

Glycogen

Immune tolerance

Transplantation

INTRODUCTION

Pompe's disease is one of the over 50 currently known lysosomal storage disorders and manifests as a metabolic myopathy due to deficiency of an enzyme named acid alpha-glucosidase (GAA). The enzyme deficiency leads to intralysosomal accumulation of glycogen and the disease is characterized by progressive loss of function of skeletal and respiratory function.

The clinical spectrum is broad. The age of onset varies from shortly after birth to presentation of first symptoms in the 6th to 7th decade of life. Patients with the most severe classic-infantile form of Pompe's disease display a profound myopathy and cardiac hypertrophy at birth or shortly later. Without treatment, these patients die within the first year of life. Other patients who usually present symptoms later in life are collectively considered as patients having non-classic forms of Pompe's disease. They do not have cardiac symptoms as characteristically seen in classic-infantile Pompe's disease. Their major symptoms are related to skeletal muscle weakness and respiratory insufficiency.

Since 2006, enzyme replacement therapy (ERT) is available for all patients with classic and non-classic forms of Pompe's disease. The most striking effect of ERT is the quick and dramatic improvement of cardiac function in classic-infantile Pompe's disease; it is life saving for these infants¹⁻⁴. Patients with non-classic forms of Pompe's disease benefit from ERT by improvement or stabilization of muscle strength and pulmonary function⁵⁻⁷. Over ten years of experience with ERT has demonstrated the need of early intervention and the difficulty to reverse existing muscle pathology^{8,9}. This has led to an important question: at which stage of disease is treatment of Pompe's disease still beneficial?

In this study we have applied hematopoietic stem cell mediated lentiviral based gene therapy at different ages in Pompe mice to investigate at what stage of disease the progressive muscle pathology can still be prevented or reversed.

At the same time, we have evaluated the effect of stem cell gene therapy aiming at one lentiviral integration per genome as a following-up of our successful attempts with multiple integration sites¹⁰. In our present studies, we did not achieve biochemical rescue of the Pompe's disease phenotype in all the tissues that we investigated even if juvenile animals were treated. In older animals the lysosomal glycogen reduction was limited to heart and liver, while the skeletal muscle did not respond significantly.

MATERIALS AND METHODS

Animals

The Pompe mouse model (FVB/N background) with complete acid alpha-glucosidase (*Gaa*) deficiency was generated as previously described¹¹. Age-matched female FVB/N mice were used as controls (obtained from Charles River Nederland (Maastricht, the Netherlands)). All procedures were approved by the animal ethical committee of the Erasmus University Medical Center, Rotterdam in accordance with legislation in the Netherlands.

Preparation of the lentiviral vector

The lentiviral vector containing the human codon optimized *GAA* cDNA sequence, pRRL.PPT.SFFV.GAAco.WPRE*.SIN was prepared as previously described (Chapter 3, this thesis). Briefly, 293T cells were transfected with 4 plasmids; one harboring the *GAA* cDNA and the other three harboring either pRSV-REV, pMDL-g/pRRE, or pMD2-VSVg^{12,13}. From day 3 till day 4 after transfection, the supernatant was collected and the vector was concentrated by ultracentrifugation. Lentiviral stocks were resuspended in PBS and aliquots were stored at -80°C until use. Lentiviral titers were calculated by titration of the lentiviral vector batch on HeLa cells and analyzed by q-PCR.

Transduction and transplantation of murine HSCs

Male *Gaa*^{-/-} mice (8-12 weeks old) were used as bone marrow donor. The lineage negative (Lin⁻) cells were purified using the mouse hematopoietic stem cell isolation Kit (BD Biosciences). After an overnight transduction in Dulbecco's medium, supplemented with murine-stem cell factor (100 ng/mL), human FMS-like tyrosine kinase 3 murine ligand (50 ng/mL), murine thrombopoietin (10 ng/mL), the transduced Lin⁻ cells were injected in the tail vein of female *Gaa*^{-/-} recipients after a sublethal conditioning regime of 6 Gy irradiation. At the time of transplant, the female recipients varied in age from 3 weeks to 52 weeks old (4-8 mice per group).

Tissue collection

The mice were fasted overnight before tissue collection at different time points after transplantation. They were sacrificed by transcardial perfusion with saline after administration of ketamine/sedator mix (Eurovet). Tissues were collected and homogenized by TissueLyserII (Qiagen), 2 times 2.5 min at 30 Hz on ice.

Biochemical assays

GAA activities and glycogen content in cell and tissue homogenates were determined as previously described^{14,15} and expressed per mg protein content (BCA kit, Pierce).

Determination of lentiviral integrations per genome

DNA from bone marrow was extracted with the NucleoSpin Tissue kit (Bioké) to determine the number of integrations per cell by quantitative PCR (q-PCR). The following primers were used: HIV-U3 forward primer, 5'-CTGGAAGGGCTAATCACTC-3', and HIV reverse primer, 5'-GGTTCCCTTCGCTTTCAG-3'. To determine the chimerism in bone marrow samples, the following primer sets were used specific for the Sry locus on the mouse Y chromosome: forward primer, 5'-CATCGGAGGGCTAAAGTGTCAC-3', and reverse primer, 5'-TGGCATGTGGTTCCTGTCC-3'. Samples were normalized to mouse *Gapdh* (forward primer, 5'-CATCACTGCCACCCAGAAGAC-3', and reverse primer, 5'-TGACCTTGCCACAGCCTTG-3') and analyzed with SDS2.2.2 software (Applied Biosystems).

Rotarod

All mice were tested on the accelerating rotarod (4 to 40 rpm in 5 minutes; Panlab/Harvard Apparatus Rota Rod). Prior to the runs, the mice were given 3 training sessions to adjust to the rotarod. The average of 3 runs with an interval of 5 minutes was calculated.

Statistical analysis

Statistical analysis was performed with Graph Pad Prism (GraphPad Software Inc., La Jolla, CA, USA). Significance of differences was determined by one-way analysis of variance or Mann-Whitney U test for comparing two groups. For repeated measurements two-way ANOVA was used to determine differences between groups. Significance of a difference was assumed when the p-value <0.05. Error bars represent SEM.

RESULTS

Hematopoietic stem cell mediated lentiviral gene therapy with minimal risk of insertional mutagenesis

Aiming at a minimal risk of insertional mutagenesis, bone marrow cells were transfected to obtain an average of 1 integration site per genome. Table 1 shows that this procedure resulted in an average of 1.41 (± 0.54) integrations of the lentiviral vector per mouse genome. The percentage chimerism was 26.31% (± 8.10) as a result of hematopoietic stem cell transplantation at the age of either 10, 25, or 40 weeks (Table 1). One copy per genome would be the ideal situation for clinical application of lentiviral based gene therapy to minimize the risk of vector related insertional mutagenesis.

Intralysosomal glycogen storage in different tissues

Untreated *Gaa*^{-/-} animals were sacrificed at 10 weeks, 25 weeks, 40 weeks and 52 weeks of age to determine the glycogen storage at that particular time. Over time we observed a continuous progression of lysosomal glycogen accumulation in heart and brain (Figure 1), whereas in diaphragm and liver the glycogen accumulated profoundly between weeks 25 and 40. In skeletal muscle, the intralysosomal glycogen accumulation reached a plateau after 10 weeks of age and stabilized around 350 µg /mg protein in the tibialis anterior (TA), 280 µg /mg protein the quadriceps femoris (quad), and 220 µg /mg protein the gastrocnemius (gastr).

Effect of stem cell based lentiviral gene therapy on glycogen reduction in organs

Previously, we have shown that complete reduction of glycogen can be achieved, even in skeletal muscle, using hematopoietic stem cell mediated lentiviral based gene therapy in mice with Pompe's disease. Very high GAA expression was achieved by the use of a codon-optimized GAA but with multiple integration sites (Chapter 3, this thesis). In those studies we could demonstrate an amelioration of the phenotype in *Gaa*^{-/-} mice

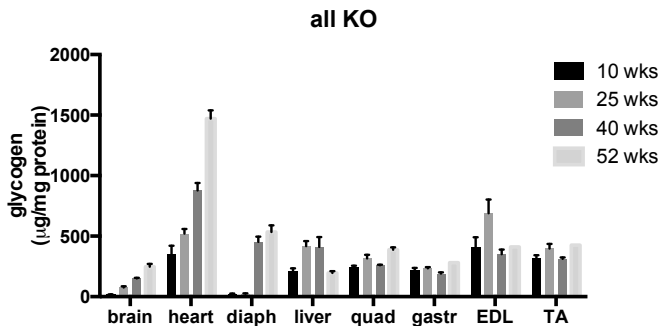


Figure 1: Intralysosomal glycogen storage in time.

The glycogen content was measured in different tissues at different ages. Diaph, diaphragm; quad, quadriceps femoris; gastr, gastrocnemius; EDL, extensor digitorum longus; TA, tibialis anterior.

Table 1: GAA activity, vector copy number and chimerism obtained in various transplantation experiments performed at different ages. Averages are shown for each age group with SEM between brackets. *Determined in spleen cells. ND: not done.

Age at transplant	GAA activity (nmol/mg/hr)	Vector copy number (VCN)	Chimerism
3 wks	14.70 (± 2.63)*	1.56 (± 0.66)*	ND
10 wks	35.65 (± 4.41)	0.89 (± 0.44)	25.42% (± 9.23%)
25 wks	42.87 (± 10.02)	1.11 (± 0.70)	26.10% (± 8.31%)
40 wks	54.33 (± 6.04)	2.10 (± 1.62)	27.77% (± 9.72%)
52 wks	ND	ND	ND

when the animals were treated at 6-8 weeks of age and sacrificed around 10 months after transplantation¹⁰. In this study, aiming at an integration of only one lentiviral copy per mouse genome, we decided to investigate whether the glycogen storage could be prevented by treating 3 weeks old *Gaa*^{-/-} mice that do not display a profound glycogen storage yet, and whether glycogen storage could be reversed by starting treatment above the age of 6-8 weeks¹¹.

The animals treated at 3 weeks of age were sacrificed at 6 months after transplantation. This group of mice with very little glycogen storage before treatment did not show significant responses (except for the liver that responded well to the therapy); glycogen levels were still very high (Figure 2), compared to untreated mice 25 weeks (Figure 3, middle panel).

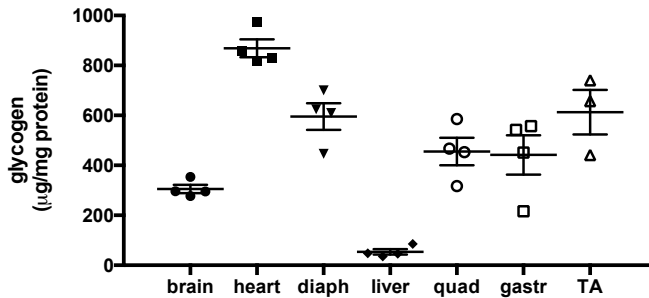
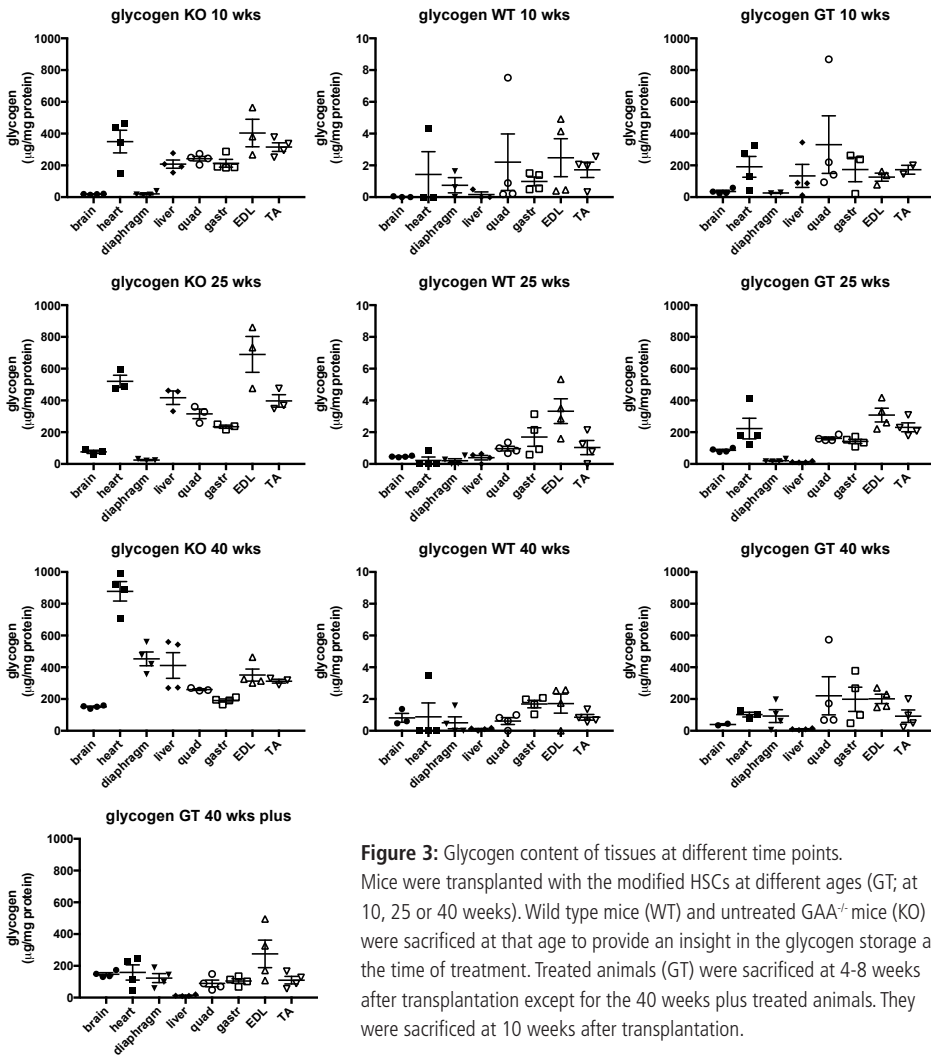


Figure 2: Effect of HSC mediated lentiviral based gene therapy performed in 3 weeks old *GAA*^{-/-} mice. The glycogen content of various organs was analyzed at 6 months after transplantation and is elevated in all tissues (normal ranges of glycogen in tissues of normal mice at 6 months of age are in the range of 0.2-5µg/mg protein).

The mice that were treated at 10 weeks, 25 weeks, 40 weeks and 52 weeks of age were sacrificed within 4-10 weeks after transplantation to minimize the risk of age-related mortality. Significant reduction of glycogen accumulation in heart and liver was observed in the three younger age groups (Figure 3), but the glycogen reduction in the 52 weeks old mice was restricted to the liver (Figure 4).

With respect to the glycogen clearance in the heart, we also measured improvement of the left ventricular mass (LVM) in mice treated at the age of 52 weeks (Figure 5). A response on the LVM was seen in these older mice, despite the fact that the glycogen content of the heart did not decrease significantly.



Effect of stem cell based lentiviral gene therapy on glycogen reduction in skeletal muscle

The mice that were treated at 3 weeks of age still had significant glycogen storage in the skeletal muscle tissues 6 months after transplantation. Treatment at 10 weeks, 25 weeks, 40 weeks and 52 weeks of age also did not result in significant reduction of glycogen storage in skeletal muscle as shown in Figures 3 and 4.

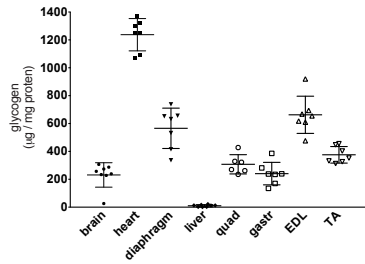


Figure 4: Effect of HSC mediated lentiviral vector based gene therapy in one year old mice. No treatment effects on glycogen storage were observed in tissues of animals treated with HSC gene therapy at one year of age and sacrificed 10 weeks later. (Average glycogen storage in untreated KO mice of same age: brain 300µg/mg protein; heart 1000µg/mg protein; diaphragm 440µg/mg protein; liver 110µg/mg protein; quad 300µg/mg protein; gastr 320 µg/mg protein; EDL 320µg/mg protein and TA around 400µg/mg protein).

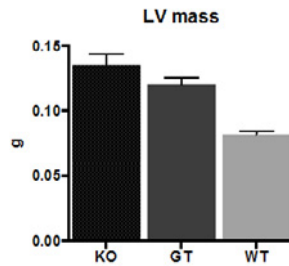


Figure 5: Effect of HSC mediated lentiviral vector based gene therapy in one year old mice on the left ventricular mass. Ten weeks after transplantation, the left ventricular mass of transplanted animals (GT) seemed slightly reduced compared with that of age-matched mock-treated *Gaa*^{-/-} knock-out mice (KO). WT; untreated wild type mice.

DISCUSSION

Treatment of *Gaa*^{-/-} mice with stem cell mediated lentiviral based gene therapy has proven to be of great potential¹⁰. We have previously shown a near complete reduction of the accumulated glycogen with this strategy, even in skeletal muscle. In these former experiments, the mice were 6-8 weeks old at time of treatment and already had glycogen storage in all tissues. Furthermore, very high multiplicity of infection (MOI) was used resulting in ~7 integrations per genome and a sustained very high expression of the GAA (Chapter 3, this thesis).

To fine-tune the procedure, we wanted to investigate whether one copy per hematopoietic stem cell would be sufficient to rescue the Pompe phenotype in the available mouse model with the optimized transgene. Secondly, we wanted to investigate the impact of hematopoietic stem cell mediated lentiviral based gene therapy when applied at different stages of disease progression.

The use of integrating retroviral vectors can lead to unwanted side effects, for instance if they occur near proto-oncogenes^{16,17}. The vector that we used is a self-inactivating third

generation lentiviral vector, which has shown to be much safer in terms of preference for sites of integrations; it shows a fairly random integration pattern as compared to the gammaretroviral vectors used in the past^{18,19}. Ideally, one would like to have as few integrations as possible without losing therapeutic benefit.

In the experiments described here, we aimed for a single integration per cell but this turned out to be insufficient to reverse the lysosomal glycogen storage in skeletal muscle of mice older than 3 weeks of age at start of treatment.

Despite the lack of effect in skeletal muscle, we did observe a significant response of the heart in animals treated at 25 weeks, 40 weeks and older than 40 weeks. The glycogen content decreased, and the LVM decreased as well. Also in humans, the heart is more responsive to ERT than skeletal muscle. One reason could be that the heart is better vascularized than the muscle and thereby more easily accessible for the circulating enzymes. Another possibility can be that one integration per cell does not provide enough GAA expression to efficiently reach the skeletal muscle. The heart requires only a minimal level of GAA activity (less than 2% of average normal) to prevent lysosomal glycogen storage in Pompe's disease, whereas more than 25% of average normal activity is required to maintain skeletal muscle function.

In previous experiments we had an average integration of ~7 lentiviral vector copies per cell, a very high GAA expression was measured in the tissues of the mice after one year follow-up and complete clearance of glycogen was obtained, even skeletal muscle weakness was prevented (Chapter 3, this thesis).

The liver responds very well in all age groups despite the low level of GAA expression. The brain, in contrast, does not respond in any of the age groups and glycogen storage in the brain is not prevented even when treatment is started at 3 weeks of age.

The fact that no optimal therapeutic effect on skeletal muscle was achieved with one lentiviral integration per cell can have several reasons. It might be that the follow-up after transplantation has been too short to obtain a maximal effect on glycogen clearance. However, engraftment of HSCs in the mouse occurs within 2-3 weeks after transplantation, while the animals were sacrificed at 4-10 weeks after transplantation. Inefficient homing of hematopoietic stem cells in older animals can be an issue. Stromal cells in the bone marrow secrete growth factors to attract the transplanted bone marrow cells from the peripheral blood and keep them inside the bone marrow^{20,21}. With age, these stromal cells excrete less growth factors and initiate less cell contacts that are important to maintain the bone marrow niche. These factors can play a role in the success of engraftment, but once engrafted the rate of HSCs proliferation is not different in young versus old recipients. We

have confirmed this in our experiments by the finding of similar percentages of chimerism in the different age groups (~26.3%).

Other attempts to achieve correction of glycogen storage in older *Gaa*^{-/-} mice with gene therapy did not result in complete glycogen reduction either. Systemically administered adeno-associated viral vectors containing the human GAA cDNA have been used in 12 and 17-months old *Gaa*^{-/-} mice but did not result in glycogen clearance in skeletal muscle²². Similar as we experienced with our lentiviral based gene therapy, the heart responded better than the skeletal muscle to the AAV mediated gene therapy.

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For future application of the proposed stem cell mediated lentiviral based gene therapy in Pompe's disease important conclusions can be drawn. One lentiviral integration per cell may be sufficient to prevent or delay disease development in very early stages of Pompe's disease, but aiming at one integration is not realistic in case of advanced muscle pathology, although the heart responds well. It appeared difficult to prevent or reverse glycogen storage in skeletal muscle. This holds for gene therapeutic approaches and ERT alike. Early start of treatment is crucial. Delayed treatment of Pompe's disease will cause irreversible muscle damage, as demonstrated in patients receiving ERT at an advanced stage of the disease^{3,23,24}. The challenges lie within finding the right balance between the risks and benefits of gene therapy and its application in Pompe's disease.

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ABSTRACT

Skeletal muscle has an impressive regenerative potential. The cells that mediate muscle repair have unique properties that are not restricted solely to the formation of new muscle, but also contribute to the repair of damaged residual tissue. Recent studies have shown that freshly isolated muscle-regenerative cells maintain these properties, and contribute to muscle repair after transplantation to host muscle tissue. Muscle-regenerative cells are typically present in low numbers, and the yield of therapeutic cells from biopsies is low. *Ex-vivo* expansion of the candidate cells is therefore required. However, when cultured *in vitro*, the muscle-regenerative cells, and particularly muscle satellite cells, lose their regenerative capacities. This poses a major limitation on the introduction of cell-based therapies for muscle disorders. Here, we take the opportunity to review the promise of cell-based therapies specifically for the treatment of degenerative muscle diseases. We focus particularly on optimizing the conditions for expanding the cells *in vitro* in a way that maintains their regenerative properties.

INTRODUCTION

Muscle disorders are a group of inherited or acquired diseases with a great variety of disease manifestations. Their common denominator is progressive loss of muscle structure and function, for which no sufficient therapy is currently available.

Cell transplantation and stem-cell-based therapies are emerging therapies. Cell-based therapies were established with the use of bone-marrow transplantations, which were performed for the first time in 1968¹. Various clinical trials (<http://clinicaltrials.gov>) have studied the potential of allogeneic stem cells such as mesenchymal stem cells, human embryonic stem-cell (hESC)-derived stem cells and hematopoietic stem cells for treating a range of conditions. Particularly exciting in this respect is the first in-man clinical trial to evaluate neural stem cells for use in patients who have suffered a stroke (a study by ReNeuron; <http://www.reneuron.com>), which is currently in progress. These developments indicate that the field of cell-based therapies is expanding and that expectations are high.

Cell-based therapies have been considered for the treatment of muscular dystrophies ever since the injection of myoblasts into a mouse model for Duchenne Muscular Dystrophy (MDX mice) resulted in the generation of dystrophin positive myofibers²⁻⁴. The initial excitement was dampened by observations that, due to poor survival, immune rejection and the limited bio-distribution of transplanted cells, the regenerative effects were both modest and transient. The field was re-ignited by the identification of muscle-regenerative cells other than myoblasts – i.e. satellite cells, pericytes and muscle-derived stem cells – with superior engraftment potential. Recent studies in animal models have shown that, upon transplantation, several muscle stem-cell populations do indeed retain their unique regenerative properties⁵⁻⁷. This sets the scene for their clinical exploration. The potential of some of the muscle-regenerative cells such as mesangioblasts is currently being evaluated in clinical trials – a development that indicates the progress in the field. Several recent reviews have extensively evaluated the properties of the different muscle-regenerative cell types⁸⁻¹¹.

The inherited muscle disorders show general involvement of skeletal muscles, often with a limb-girdle distribution, indicating both that several muscle groups need to be targeted and that considerable numbers of donor cells are required. It remains an important practical limitation that the candidate populations can often be obtained only in small numbers, and that expansion of these populations is required to obtain these cells in clinically relevant numbers. A potential advantage of using cultured therapeutic cells is that they might offer an opportunity to correct the disease-causing genetic defect before injection, potentially opening the way for the development of an autologous cell-based therapeutic approach. Furthermore, autologous stem/regenerative cells will greatly reduce the risk of immune rejection that limited the success of earlier muscle-cell transplantation strategies. However, two major concerns are associated with the culture of therapeutic cells: the loss

of regenerative potential and the acquisition of genomic instability. If the development of cell-based therapies is to be successful, these considerations should be taken into account.

In this review, we highlight several types of muscle-regenerative cells with distinct properties and focus on recent approaches and advances in the expansion of muscle stem or progenitor cells. The systematic application of these strategies will be essential to exploring the further clinical application of these exciting new treatment modalities.

SKELETAL MUSCLE DISORDERS AND MUSCLE REGENERATION

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Muscle disorders

Skeletal muscle comprises the body's largest tissue, accounting for about 40% of total body weight, and playing critical roles in movement, respiration, stabilization of the skeleton, glucose homeostasis, and thermoregulation. It consists of bundles of multinucleated, elongated membrane-bound cells, called muscle fibers. These fibers contain bundles of myofibrils showing a striated pattern of repeating units, known as sarcomeres, which are the fundamental contractile units of skeletal muscle. The myofibrils set off a mechanical contraction in response to neuronal or electrical stimuli, generating the contractile force needed by a particular skeletal muscle to perform its function. Adult skeletal muscle also houses several populations of stem cells, which play important roles in maintaining the integrity of the tissue and mediating the repair of any damage to the muscle. As we discuss below, satellite cells comprise the predominant muscle stem cell population responsible for postnatal muscle regeneration¹².

Acute or chronic muscle damage results from the disruption of the structural organization of the muscle, inducing muscle-fiber necrosis, infiltration of inflammatory cells, and the deposition of non-myogenic material (e.g. connective tissue, fat, and glycogen deposits). Many hereditary and acquired neuromuscular disorders – including the muscular dystrophies, toxic, inflammatory and metabolic myopathies, and neuropathies leading to muscle denervation – are associated with muscle damage. Muscle damage is also seen in systemic conditions such as ageing, cancer and endocrinological disorders. The neuromuscular disorders are a heterogeneous group of rare disorders that may present at any age and may significantly reduce life-expectancy, especially when the cardiac and respiratory muscles are involved (such as in Duchenne Muscular Dystrophy (DMD) and Pompe's Disease).

Muscle disorders are associated with a lengthening list of defects in genes that encode cytoskeletal, lysosomal, sarcomeric and membrane-associated proteins. The clinical and pathophysiological hallmarks of these myopathies can vary widely and are beyond the scope of this review (interested readers are referred to the specific literature, see for instance¹³⁻¹⁵). However, irrespective of the mechanisms involved, the common denominator is a muscle-wasting phenotype. With regard to several of the inherited muscular dystro-

phies (Table 1), it is thought that disease progression is determined largely by exhaustion of the stem-cell pool and the resulting progressive loss of muscle-regeneration potential. On the basis of this assumption, it is possible that the attenuation of muscle damage or the restoration of the muscle-regenerative potential is key to the effective treatment of neuromuscular disorders.

Muscle regeneration in healthy and diseased muscle

Minor damage to the muscle fibers is patched by the family of dysferlin proteins ¹⁶, while more extensive injury results in the activation of muscle-resident stem cells that marks the initiation of the regenerative response. Recent studies have shown that adult muscle regeneration depends mainly on one population of stem cells, the satellite cells (SCs; Figure. 1) ¹⁷⁻¹⁹. Upon sustaining damage, the activated SCs start to proliferate and generate committed myoblasts, which differentiate into myocytes. To repair the damage, these myocytes fuse with each other to make new myofibers, or fuse with the residual myofiber (Figure 1).

While the repair process in healthy muscle is completed within one to three weeks, depending on the extent of the damage, the regenerated muscle fibers of dystrophic muscle remain unstable due to the underlying genetic defects, and continue to accumulate damage. As a result, dystrophic muscle engages in continuous rounds of degeneration and SC activation. These ongoing cycles of muscle degeneration and regeneration characterize dystrophic muscle, and are thought to result in exhaustion of the SC pool (discussed further below) which progresses to loss of function of the affected muscle and, eventually, to muscle atrophy. It is unclear whether ongoing muscle regeneration occurs during the disease progression of all muscle disorders (such as facioscapulohumeral dystrophy; FSHD). Even for conditions in which mainly atrophy has been observed (and loss of SCs is not implied), the affected muscles will have reduced regenerative potential, and muscle wasting will be progressive.

It has been proposed that muscle is capable of complete regeneration when SC numbers are at least 10-20% of those in young adults ²⁰. This may indicate that the numbers of regenerative-competent SCs decrease below the critical threshold during disease progression, which may imply that even a modest increase in stem-cell numbers would have a beneficial effect in diseased muscle. This observation may provide a basis for cell therapy of muscle disorders using muscle-regenerative cells.

Mechanisms of satellite-cell exhaustion

Postnatal muscle growth and regeneration is mediated by muscle satellite cells, which characteristically reside beneath the basal lamina and were first described over fifty years ago by Alexander Mauro ²¹. Recently, to celebrate their discovery, excellent reviews on them ^{12,22} were published. SCs are characterized by the expression of the paired box transcription factor Pax7 across species, including man, mouse and chicken ²². Several recent studies

Table 1: Muscle disorders with indication of SC exhaustion.

The table lists a number of muscle disorders with indications of satellite cell (SC) exhaustion. SC exhaustion may be direct if the gene is expressed in SC pool and affects the function of SCs. The effect of the gene defect may be indirect if the gene is normally not expressed by SCs. The disease causing genes and relevant animal models are listed.

Disease	Gene ¹	Animal model ²	SC exhaustion ³
<i>Inherited muscular dystrophies</i>			
Becker Muscular Dystrophy	Dystrophin (Xp21)	MDX mouse [131], mild phenotype	Indirect: Functional change: extensive activation; change in environment [25]
Congenital Muscular Disorders	Laminin A2/Merosin (6q22-6q23)	e.g. Laminin A2-deficient mouse [132]	
	Integrin A7 (12.q13.2)	Integrin A7-deficient mouse [133]	Indirect? Changes in environment (loss of Integrin A7) [150]
	Fukutin (9q31-q33)	Fukutin chimeric mouse [134]	
	SEPN1 (1p36)	SEPN1-deficient mouse [135]	Direct: increased proliferation SCs [151]
Duchenne Muscular Dystrophy	Dystrophin (Xp21)	MDX mouse [131]; GRMD dogs [136]	Indirect: Functional change: extensive activation; change in environment [25]
Emery-Dreifuss	Emerin (Xq28)	Emerin-deficient mouse [137]	Direct: Premature differentiation/cell cycle exit [24]
	Lamin A/C (1q11-q21)	Lamin-A-deficient mouse [138]	
Fascioscapulohumeral Muscular Dystrophy	FSHMD1A (95%;4q35)	FRG-1 transgenic mouse [139]	Direct and indirect: Proliferation/differentiation defect myoblast [39,40]
Limb-Girdle Muscular Dystrophy	Dysferlin (2q13.2)	Dysferlin-deficient mouse [140]	Indirect: Inhibition of myoblast fusion [154]
	e.g. Alpha-sarcoglycan (17q12-21.33)	BIO 14.6 hamster [141]	
	POMT1 (9q34.1) and POMT2 (14q24.3)	POMT1-deficiency in mouse is embryonic lethal [142]	Indirect: apoptosis in drosophila myoblasts [155]
	DMPK (DM1; 19q13.2-q13.3)	DMPK-deficient mice [143]	Indirect: Myoblast dysfunction was reported [156]
	ZNF9 (DM2; 3q21)	ZNF9+/- mouse [144]	
	PABPN1 (14q)	Transgenic mouse expressing mutated PABN1 [145]	Direct and indirect: defects in myoblast differentiation and proliferation [157]
<i>Metabolic Myopathies</i>			
Late-onset Pompe Disease	Acid alpha-glucosidase (17q25.2-q25.3)	GAAGO mouse [146,147]	Unknown: Increased SC activation reported [158]
<i>Acquired Myopathies</i>			
Stress Urinary Incontinence ⁴	Ageing	Models reviewed by [148]	Indirect: Age-related loss of replicative potential, apoptosis [31]
Chronic Obstructive Pulmonary Disease (COPD)	Alpha-1 Antitrypsin (14q32.1)	Klotho knockout [149]	Indirect: Replicative senescence/reduction minimal telomere length [33]

1=Genes involved in disease; human gene locus between brackets, for some syndromes more genes were implicated and are indicated in a new row

2=Examples of animal models, mainly mouse, are listed. The selected models were reported to give a relevant phenotype, unless indicated otherwise. References are shown between brackets

3=Exhaustion of SC pool may be direct (gene expressed and has critical function in SC) or indirect (gene not expressed in SC). Adapted from [159]; Relevant references for SC exhaustion are shown between brackets

have shown that SCs are *bona-fide* stem cells²³ and generate both differentiating progeny (myoblasts and myocytes) and, in a process called self-renewal, new SCs. As stated above, muscle regeneration does not proceed in the absence of SCs¹⁷⁻¹⁹ and loss of SC numbers or activity is thought to be at the basis of the muscle-wasting that is observed in the diverse conditions affecting skeletal muscle. There may be several mechanisms, both cell-intrinsic and -extrinsic, underlying SC exhaustion in dystrophic muscle and here we will discuss several of the mechanisms that have been proposed.

In some hereditary myopathies, the association between the gene defect and SC exhaustion is very clear. In these cases, the 'disease' gene is normally expressed in SCs in healthy individuals; the absence or loss of function of this gene directly affects SC function. For instance, lamin A/C deficiency in Emery-Dreifuss myopathies induces premature SC differentiation and cell-cycle exit²⁴ (Table 1). As a result, the SC pool is depleted and muscle-regenerative potential progressively lost.

Other disease-causing genes, such as dystrophin, are not expressed in the SC compartment, but only in the terminally differentiated myofibers. While loss of function of these genes is directly related to myofiber stress and damage, the lack of expression of these genes does not directly affect SC behavior. In these cases, the progressive muscle-wasting alters the architecture of the muscle in a process that may involve inflammation, fibrosis or deposition of non-myogenic material, as has been described for DMD^{25,26}. As SCs reside in a specialized niche formed by the basal lamina covering them, their functioning and survival is dependent on the availability of this niche. The detrimental changes to the muscle architecture inhibit the potential of SCs to regenerate the muscle, or even induce the death of SCs.

The importance of the SC environment in determining the muscle regenerative response has also become clear from heterochronic transplantation studies. These studies showed that the age of the host determines the efficiency of the muscle-regenerative response²⁷. Aged muscle progenitors were capable of efficient muscle regeneration when transplanted into a young host. More recent findings using a heterochronic parabiosis approach further substantiated these early findings and showed that circulating factors play key roles in determining the regenerative potential of aged SCs²⁷⁻³⁰. The progressive apoptosis of SCs observed in stress urinary incontinence (SUI), which is an age-related myopathy, has been proposed to result from a changing (ageing) environment³¹ and would be supporting the findings in the (parabiotic) mouse studies. In conclusion, changes in the availability of 'regenerative factors' in the aged or diseased environment (either niche or circulating factors) limit an adequate regenerative response of SCs and contribute to functional and numerical loss of SCs.

As a third mechanism of SC exhaustion excessive activation/proliferation of the SC pool has been proposed, such as for instance in DMD³² and chronic obstructive pulmonary

disease (COPD) ³³ (Table 1). As stated above, some dystrophies and muscle-wasting conditions are characterized by continuous cycles of degeneration and regeneration, and lead to excessive use of muscle SCs. This is thought to induce replicative stress and is attributed to telomere erosion ^{34,35} or oxidative stress ³⁶. As a result, the SC pool becomes progressively depleted. The association of replicative stress by telomere erosion with the dystrophic phenotype is underscored by findings in the mouse model of Duchenne Muscular Dystrophy, the MDX model. Relative to human patients, MDX animals have a very mild phenotype and a near-normal lifespan. However, using MDX/mTRnull (mTR is the telomerase RNA component Terc) compound mouse model, the authors showed that loss of telomere maintenance exacerbated the phenotype of the MDX mouse, more closely mimicking the disease progression observed in human patients. The study of Sacco et al. found the function of the SC population to be compromised and the SC pool to become depleted with disease progression. Myoblasts isolated from the MDX/mTRnull mice were also found to have significantly shorter telomeres. Taken together, these findings suggest that loss of telomere length and replicative stress contributes to the muscle pathology in DMD ³⁵. A recent study on a modest number of patients found reduced minimal telomere length in limb muscle in COPD patients, resulting in an exhausted muscle regenerative capacity and a muscle-wasting phenotype ³³. This indicates that exhaustion of the SC pool through excessive proliferation is not only restricted to DMD, but may contribute to loss of muscle function and mass in other muscle disorders as well.

CELL-BASED THERAPIES

Therapy of muscle disorders

There are currently few treatment options for muscle disorders. One of the few myopathies for which a relatively effective treatment modality is available is Pompe's disease. Patients with this disease develop skeletal muscle pathology due to storage of glycogen in the lysosomes caused by acid α -glucosidase (GAA) deficiency. The clinical symptoms of Pompe's disease can manifest at any age ³⁷. Patients of all ages receive enzyme replacement therapy (ERT). The rapid demise of infants with symptoms presenting at birth is prevented by correction of their cardiac hypertrophy and by the maintenance of their pulmonary function. Most treated infants acquire sitting and walking abilities while they would have had a life expectancy of less than 1 year if untreated ³⁸. Patients with later onset and less progressive forms of Pompe's disease benefit from enzyme replacement therapy and show improved walking capacity and stabilization of pulmonary function ³⁹. Recent results suggest that ERT in these patients also prolongs survival (Gungor/ van der Ploeg, *personal communication*). Despite the success of this treatment, a number of limitations are associated with

ERT –including poor responder patients, development of resistance to ERT and the high treatment costs – thus explaining the need for novel treatments.

For most of the other muscle disorders, no treatments are currently available and most approaches offer palliative care. However, some of the treatments that are in use, such as for instance glucocorticoid treatment for DMD, actually attenuate disease progression. Glucocorticoid treatment slows down the loss of muscle strength, prolongs ambulation, and supports respiration⁴⁰ and even though suboptimal is currently the standard treatment for DMD⁴¹.

Some experimental therapies (such as exon skipping for DMD⁴² (e.g. clinical trial identifier NCT00159250)) have reached the clinical trial phase, and the hope of a positive outcome is high. Inherited muscular diseases are promising targets for gene-therapy strategies: in most cases, the etiology of the disease involves a single gene (so-called single-gene disorders).

Cell therapy for the treatment of muscle disorders is one alternative being considered as an alternative to ERT, gene therapy or other experimental approaches. Its promise is discussed below.

Rationale for the use of muscle stem cells in the treatment of muscle disorders

Cell-based therapies are particularly promising for the treatment of muscle disorders, as they would enable the robust regenerative properties of muscle-regenerative cells to be exploited. Muscle-regenerative cells are attractive for therapy for three reasons: their ability to generate new myofibers, to repair damaged myofibers, and to correct the genetic defect through cellular fusion. The ability to fuse and share genetic material with the regenerated myofibers is an inherent programmed activity of muscle-regenerative cells and is restricted to muscle regeneration. When cells from healthy donors are used, or when gene-corrected autologous cells are used, this property can be employed to restore expression of the disease-causing genes.

In addition to these properties, most of the cell types that are considered for muscle-regenerative purposes (see below) replenish the stem-cell pool. As discussed above, exhaustion of the endogenous stem cell pool is thought to contribute to the muscle-wasting phenotype that is common to a subset of muscle disorders. The self-renewing transplanted cells continue to be recruited during ongoing cycles of regeneration and expand the regenerated area. Over time, the condition of the transplanted muscles improves, potentially restoring the function of the affected muscles.

Based on these properties, we and others hypothesize that the use of cells with myogenic potential may make it possible to arrest or attenuate the muscle-wasting process that is common to all myopathies.

Several donor cell types as a source of muscle stem-cell therapy

Skeletal muscle is known to harbor several populations of stem cells, including satellite cells^{21,23}, interstitial cells⁴³ and vessel-associated cells⁴⁴, and novel candidates continue to be identified. The predominant muscle-resident stem cells are the satellite cells (SCs), which recent independent studies have shown to be mainly responsible for postnatal muscle growth and regeneration¹⁷⁻¹⁹. It is still unclear whether the non-SC populations are involved in physiological and pathophysiological muscle repair in the adult muscle, and, if so, which role they play. As vessel-associated cells have been shown to contribute to the SC pool early in postnatal life⁶, it has been suggested that a subset of the non-SC population are SC progenitors⁴⁵. But as SC populations^{5,7} and non-SC populations^{43,44} both display potential to regenerate muscle and replenish the endogenous SC pool upon transplantation^{6,46,47}, both qualify as significant candidate donor-cell populations for cell therapy.

The various muscle-regenerative cells have different properties, and ideally the candidate donor cells should comply with the following features:

- They should have a robust muscle-regenerative potential
- The cells should have the potential to expand *ex-vivo* while maintaining their regenerative properties
- They should contribute to the stem-cell population and replenish the SC pool
- They should induce minimal immunogenicity
- The cells should have the potential to be delivered systemically, although cells delivered locally may be clinically relevant.

We discuss these guidelines for two candidate cell populations with distinct properties: cells with the highest myogenic potential after local delivery (myoblasts/satellite cells), and cells that can regenerate muscle after systemic delivery (vessel-associated cells). The properties of other muscle-regenerative cells are summarized in Table 2.

To illustrate their clinical potential the two selected cell types are described only briefly. For a much more detailed discussion of the properties and clinical potential of the different muscle-regenerative cells readers are referred to several recent reviews^{10,11,48,49}.

Muscle stem cells for local delivery: myoblasts/satellite cells

The myogenic lineage constitutes different cell populations with distinct phenotypical and functional properties. SCs (Pax7-expressing cells in the mouse; Figure 1) are the predominant muscle-resident stem-cell population and are capable of proliferation and self-renewal. Upon activation, SCs enter the cell cycle and progress to become myoblasts (pax7-/myod+; Figure 1), which represent committed progenitors⁵⁰. Equipped with limited self-renewal, but extensive differentiation potential, myoblasts, undergo a limited number

Table 2: Regenerative properties of muscle regenerative cells. The table depicts different types of muscle regenerative cells, most of which are resident to skeletal muscle. Each type of muscle regenerative cell is scored according to the five requirements of the optimal candidate for stem cell therapy.

Donor cell type	Primary location	Muscle regeneration ¹	Ex vivo expansion with regenerative potential ²	Contribution to Stem cell pool ³	Immunogenicity ⁴	Systemic delivery	References
Bone marrow derived stem cells	bone marrow	Yes	no	yes phenotypic	unknown	yes	[160]
iPS cells	skin (fibroblasts)	yes	yes	yes phenotypic	unknown	no	[46]
Mesenchymal Stem Cell (MSC)	all organs	yes	yes at least 15 passages	no	unknown	yes	[161]
Muscle CD133+ve cells	muscle interstitium/circulation	yes	limited (blood-derived), extensive (muscle-derived)	yes phenotypic	Unknown	yes	[80, 162]
Muscle SP cells	muscle interstitium	Yes	not tested	yes functional (re-injury)	low	not studied	[163]
Muscle-Derived Stem Cells	unknown	yes	yes, MDSC were tested at passage 10-12	yes phenotypic	low	no	[64]
Myoblasts	myofiber	yes	easy to culture, loss of regenerative potential	yes (probably SC subpopulation)	high	no	[4]
Myoendothelial cells	blood vessels	yes	yes with regenerative potential	no evidence	unknown	no	[164]
PICs (PW1+ve cells)	muscle interstitium	yes	not tested	yes (phenotypic)	Unknown	not studied	[43]
Satellite Cells	sublamina	yes	no loss of regenerative potential	yes functional (serial transplantation)	low	no	[5,7]
Vessel-associated cells	blood vessels muscle	yes	early passages were tested	yes phenotypic	low	yes	[44, 55]

1=Contribution to muscle regeneration after transplantation is scored

2=Scored positive ('yes') if the studies report regeneration potential *in vivo* following extensive *ex vivo* expansion. In most cases freshly isolated cells were evaluated

3=Scored positive when functional (e.g. serial transplantations or re-injury experiments) or phenotypic contribution to the stem cell pool was reported. Contribution was scored as 'phenotypic' only if study report localization to the SC niche with/without expression of Pax7

4=Immunogenicity was considered low, when cells engrafted muscle of immunocompetent hosts

of divisions before differentiating into myocytes (Figure 1). Myocytes are differentiated muscle cells that have upregulated myogenin and are programmed to fuse either with each other (thereby forming neofibers) or with damaged myofibers.

On the basis of their extensive proliferation and differentiation potential *in vitro*, myoblasts have long been considered for muscle cell-therapy. Initially, very promising results were produced by using myoblasts as donor cells for transplantation purposes (Myoblast Transfer Therapy; MTT) ⁴, but subsequent studies revealed a number of obstacles that complicated their introduction into the clinic. These included poor survival, immune rejection, and limited migration of the donor cells ^{51,52}.

It was hypothesized that muscle stem cells, SCs, would have greater regenerative potential, and recent studies have indeed demonstrated the remarkable muscle-regenerative potential of freshly isolated SCs ^{5,7,47}, which succeeded in repopulating muscle even after transplantation of a single SC ⁷. After transplantation into muscle of MDX hosts, SCs were shown to restore dystrophin expression ⁵. This also restored interest in the therapeutic potential of myogenic cells. As well as contributing to muscle regeneration, transplanted

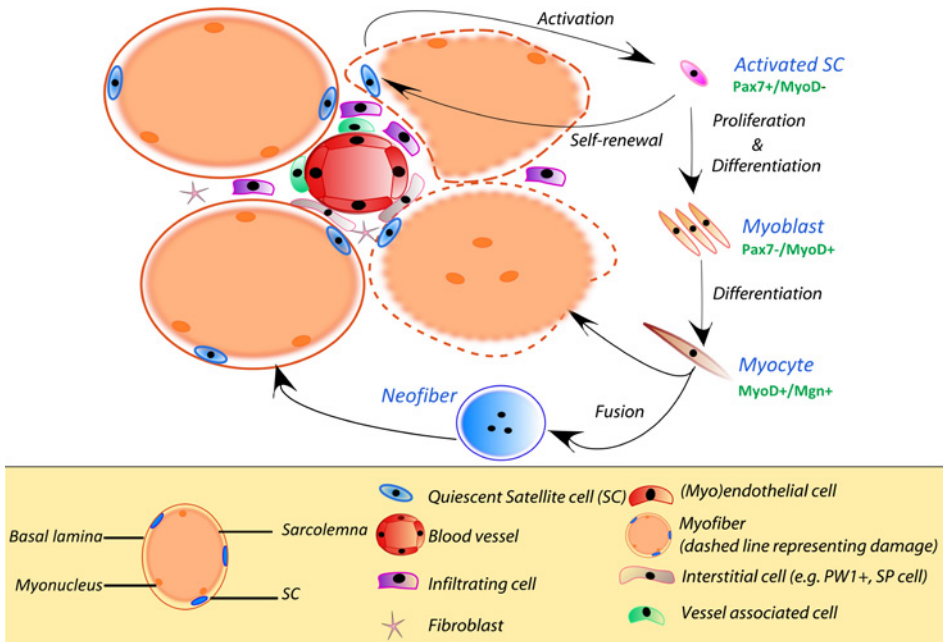


Figure 1: Skeletal muscle regeneration and muscle resident stem cells. The figure depicts a cross-section of skeletal muscle showing myofibers surrounding a blood vessel and the localization of several types of muscle-resident stem cells. The satellite cells (SCs) are located at the periphery of the myofibers, beneath the basal lamina, and become activated upon damage. The activated SCs start expressing myogenic factors, including MyoD and progress to become myoblasts. Myoblasts upregulate the expression of the differentiation factor myogenin (Mgn) and differentiate into myocytes that finally fuse together (forming neofibers) or to damaged myofibers. The figure is adapted from Kuang et al.¹¹⁴ Trends Mol Med 14: 82-91.

SCs were shown to give rise to new SCs ^{5,7,47}, indicating that SCs retain the potential to self-renew upon transplantation. Recently this self-renewal capacity was further demonstrated by a serial transplantation assay ⁴⁷, which is currently the most stringent assay for showing self-renewal potential.

Unlike myoblasts, SCs had low immunogenicity. Cerletti and colleagues have shown that healthy SCs transplanted into the muscle of immune-competent MDX mice resulted in robust donor-cell engraftment and contribution to the formation of host-donor chimaeric myofibers that lasted up to 4 months after transplantation ⁵. The authors even reported reduced inflammation of the host muscle, indicating that the transplanted cells did not generate a strong immune response.

Despite their promising regenerative potential, SCs share a major limitation: their low migratory potential. SCs and myoblasts therefore have limited or no ability to engraft after systemic delivery, while a contribution to muscle regeneration following intramuscular injection is often observed only in the proximity of the injection site. Recently, this was also verified for systemically delivered human muscle progenitors (i.e. SC-derived myoblasts) that failed to engraft in dystrophic muscle ⁴⁴. The same study showed that the human muscle progenitors contributed robustly to muscle regeneration after intramuscular injection.

Taken together, the properties of SCs, and, to a lesser extent, of myoblasts, are most suitable for the treatment of disorders affecting specific muscles, such as stress urinary incontinence (rhabdosphincter mainly affected) or oculopharyngeal dystrophy (affecting primarily the extraocular muscles).

SCs may also be used to regenerate selected muscle in systemic muscle disorders. For instance, it has been suggested, from a DMD patients' perspective, that it would be invaluable to preserve or improve the function of hand and finger muscles ⁵³. In addition, the diaphragm muscles in DMD or Pompe's disease would be attractive targets for SC-based therapy.

Muscle stem cells for systemic delivery: vessel-associated cells/Mesangioblasts/Pericytes

Currently, the most promising candidates for muscle cell-therapy are the cells isolated from the wall of blood vessels in the embryo ⁵⁴ (mesangioblasts) or in the adult (pericytes) ⁴⁴. In adults, mesangioblasts are thought to be a subset of pericytes ^{44,55}. For reasons of clarity, both these cell types are discussed here as vessel-associated cells, which can be isolated from vessels throughout the body, are multipotent, and can differentiate into different types of mesoderm. When isolated from the vessels present in muscle, these cells were shown to be robustly myogenic *in vitro* and *in vivo* ^{44,54}. Interestingly, it has been reported that, after transplantation, vessel-associated cells contribute to the SC pool ⁶; this was explained by the fact that vessel-associated cells and SCs share a common origin

in the embryo. Even in response to muscle-toxins or dystrophy, these 'vessel-associated cell-derived' satellite cells expressed Pax7, and contributed to muscle homeostasis and regeneration⁶. This may explain their ability to contribute to muscle regeneration under certain conditions, for example after transplantation to distressed muscle.

Their muscle-regenerative (and therapeutic) potential is clearly indicated by their ability to restore or ameliorate the dystrophic phenotype after transplantation to dystrophic mice (α -sarcoglycan-*null* mice⁵⁶ and dysferlin-deficient mice⁵⁷) and golden retriever muscular dystrophy dogs⁵⁵. The ability of vessel-associated cells to morphologically and functionally restore the dystrophic phenotype in α -sarcoglycan-*null* mice (the animal model for limb-girdle muscular dystrophy 2D) indicated that a robust immune response to these cells was lacking or did not limit engraftment. In line with this, vessel-associated cells from sources other than muscle are shown to have low immunogenicity^{58,59}. Given the robust immune response (and hence limited engraftment) observed after myoblast transplantation, this property may be an important attribute for the therapeutic potential of vessel associated cells.

One drawback may be, that in the absence of well-defined markers, that it has been found to be difficult to prepare pure populations of vessel-associated cells with robust reproducible regenerative potential⁶⁰. Additional cell types may contaminate the isolates and fail to contribute to regeneration, thereby affecting the experimental outcome.

Vessel-associated cells are attractive candidate for therapy due not only to their muscle-regenerative potential, but also to their ability to proliferate *in vitro*. It was reported that they could be expanded by up to 20 population-doublings before undergoing senescence. This was claimed to be sufficient to treat a young patient⁴⁴.

In conclusion, the properties of vessel-associated cells, particularly their compatibility with systemic delivery, makes these cells good candidates for treating systemic muscle disorders such as DMD and limb-girdle muscular dystrophy.

THE *EX-VIVO* EXPANSION OF REGENERATIVE CELLS

SCs comprise about ~4% of myonuclei in human adult muscle, and only limited numbers of regenerative cells can be obtained from patient muscle samples. This indicates that extensive *ex-vivo* expansion is required to increase cell numbers – and thereby the feasibility of cell-therapy. However, culturing freshly isolated (mouse) SCs and human muscle progenitors leads to the generation of committed progenitors whose regenerative potential is reduced⁶¹⁻⁶⁴. This loss of regenerative potential upon *ex-vivo* expansion is not unique to the culturing of SCs: it is also acknowledged for other types of stem cell that are used for therapy, including hematopoietic stem cells⁶⁵. Even vessel-associated cells, which

can be expanded rather extensively *ex vivo*, eventually undergo senescence, while further expansion may be required to treat adult or severely affected patients.

The need for refined culturing techniques is most apparent for SCs, and great progress has been made in understanding the mechanisms that regulate their stem-cell properties. Here we discuss various culturing techniques described in several studies, and how they may be used in future studies to expand cells with the highest regenerative potential.

Understanding the regulation of stem-cell fate

The endogenous stem-cell pool is maintained *in vivo* through the tight regulation of self-renewal and differentiation. The regulation of these processes is highly complex and is determined largely by environmental factors. The importance of the stem-cell microenvironment, or niche, has been convincingly shown for SCs. SCs are polarized cells with a basal membrane rich in $\alpha7/\beta1$ -integrin that is in direct contact with the laminin-rich basal lamina surrounding the myofibers. The apical membrane of the SC expresses M-cadherin and receives signals from the myofiber. Displacement of one SC daughter cell from the niche after dividing perpendicular to the length axis of the myofiber results in lineage commitment of the apical daughter. The basal daughter remains in the niche (defined by the basal lamina) and retains the stem-cell fate. In contrast, SCs dividing in a planar orientation generate daughter cells with identical stem-cell fates, as the dividing cells maintain contact with the basal lamina ⁶⁶.

Other indications for the dominant effect of the environment on stem-cell fate were obtained from heterochronic transplantation studies. Satellite cells' age-related loss of regenerative potential could be restored by heterochronic transplantation of aged SCs into a young environment, while the reverse transplantations were ineffective ^{27,29}. The importance of the proper environment in dictating the regenerative potential of its associated stem cells is further demonstrated by the success of intact single-myofiber transplantation in contributing to new myofibers and the generation of donor-derived SCs ^{23,67}. During the transplantation procedure the SCs remained in their natural niche in these intact myofibers, which is thought to be vital for ensuring their robust regenerative potential. The results of these studies strongly suggest that the signals for governing cell fate and regenerative potential can be identified by dissecting the SC microenvironment. The niche is composed of both soluble and solid biochemical signals (oxygen, growth factors, nutrients, cytokines, extracellular matrix proteins), and confers biophysical signals (e.g. matrix stiffness, fluidity, oxygen tension).

In addition to signals from the environment, cell-specific factors are critical, and the cell within the niche should be properly programmed to interpret the stem-cell signals. This has been shown for bone-marrow-derived cells (BMDC), which occasionally occupy the SC niche ⁶⁸. These BMDCs did not acquire a myogenic fate during their residency in the SC niche.

Furthermore, there are numerous examples where conditional targeting (e.g. inactivation) of a SC-specific gene that had no effect on the niche, resulted in activation, proliferation and often premature differentiation of SCs. For instance, a recent study targeted Myf5 mRNA expression by inactivating Mir31, which targets Myf5 in quiescent stem cells and prevents accumulation of Myf5 protein⁶⁹. Myf5 belongs to the family of muscle regulatory factors (MRFs), which also includes MyoD, MRF4 and myogenin, and is expressed in quiescent satellite cells and early muscle progenitors. After inactivating Mir31 by the intramuscular injection of specific antagomirs (chemically designed oligonucleotides used to silence Mirs), quiescent satellite cells re-entered the cell-cycle, and muscle regeneration increased; this was deduced by the presence of an increased number of small embryonic myosin heavy chain (eMHC; detected only in regenerating myofibers) positive myofibers. In addition, two recent studies showed that conditional SC-specific inactivation of RBP-J, a nuclear factor essential in Notch signaling, resulted in SC depletion and loss of muscle-regenerative potential^{70,71}, while the niche remained intact in these animals. These studies indicate that targeting of certain cell-intrinsic factors dictates cell fate, an effect that may be exploited during *ex-vivo* culturing.

Expanding or selecting subpopulations with higher regenerative potential

SC populations are phenotypically and functionally heterogeneous⁷², their regenerative potential varying between SC subpopulations. The heterogeneity in regenerative potential of SC subpopulations is maintained *ex vivo*^{47,51,73,74}, which may allow the selection and expansion of the most highly regenerating subpopulations. All one would need is to identify and trigger the proper stimuli.

A recent study took a label-retention approach to selecting the slow-dividing cell population from SC-derived muscle cultures⁷³. In several types of tissues and cultures there are indications that slowly dividing cells represent the subpopulation with increased stem-cell potential. For instance, quiescent HSC demonstrated increased survival after transplantation, while short-term culture induced cell-cycle reentry and failure to reconstitute NOD/SCID animals^{75,76}. In line with this, the slowly dividing population identified in murine SC-derived muscle cultures was shown to harbor increased myogenic potential *in vivo* and to generate a functional SC population⁷³. The dyes used for label-retaining experiments are DNA-binding chemicals, so to use this strategy for clinical purposes the safety of label will be a relevant issue.

On the basis of the hypothesis that the ALDH^{hi} population would harbor increased resistance to oxidative stress, another study selected a subpopulation of cells expressing high levels of alcohol dehydrogenase (ALDH^{hi}) from murine and human muscle cultures. Oxidative stress is thought to be one of the major factors that limited myoblast engraftment in the early myoblast transfer studies⁷⁷. The study by Vella and co-authors indicated

that stress resistance, proliferation, differentiation and muscle regeneration were increased in the ALDH^{hi} population of both species.

FACS sorting is widely used to enrich for cell populations,^{78,79} and several cell-surface markers, including CXCR4 and CD133, have been reported to allow the isolation of highly regenerative cells directly from donor muscle^{5,7,80}. These sorted subpopulations have a high regenerative potential, and it would be of clinical interest to expand them *ex vivo*. As transplantation studies have shown that only a limited number of such cells would be needed to obtain robust engraftment potential⁷, a minimal *ex-vivo* expansion may be required. Unfortunately, these FACS-sorted populations either lose their regenerative potential upon *ex-vivo* expansion⁷, or have limited potential to proliferate *in vitro*⁸⁰. So, to maintain the high level of regeneration potential, FACS-sorted populations should be cultured under optimized conditions, as will be described below (e.g. by stimulating self-renewing expansion).

Alternatively, as muscle populations remain heterogeneous in culture and harbor subpopulations with increased regenerative potential⁵¹, a FACS-sorting strategy may allow purification of engraftment-competent cells from extensively expanded muscle cultures. So far, however, no cell surface marker (s) have been identified that could be used for such a strategy, although this is currently one of the main interests in our laboratory.

Inducing self-renewing expansion

Much work has been done to understand the molecules that contribute to the self-renewal of SCs and prevent their premature differentiation. These studies have revealed important roles for soluble signaling molecules, including Notch and Wnt ligands, and also for several membrane proteins such as caveolin-1 and syndecan 3/4 (reviewed by Kuang et al.⁸¹). Most of the knowledge is derived from studies investigating this mechanism *in vivo*, but the importance of these pathways for self-renewal have been verified *in vitro*⁶³.

The importance of the Notch pathway in regulating SC behavior and size of the SC pool was shown in earlier studies where pharmacological inhibition of Notch signaling inhibited the proliferation and self-renewal potential of SCs, while the enhancement of Notch activity restored the regeneration potential of aged muscle^{28,66}. As stated above, SC-specific inhibition of Notch signaling *in vivo* by conditional inactivation of RBPJ induced premature differentiation. These Notch-inhibited SCs differentiated without first undergoing cell division and fused with adjacent fibers. As a result, the SC pool was gradually depleted^{70,71}. A similar effect was shown on embryonic muscle progenitors after deleting RBPJ⁸². In Hes1/3 double knockout mice (target genes of Notch signaling), a defect in generating undifferentiated SC was observed and SC numbers decreased gradually⁸³. On the other hand, constitutive Notch activation *in vivo* increased Pax7 expression and promoted SC self-renewal⁵⁰.

Notch activity was shown to also determine self-renewal and increase the number of undifferentiated SCs (Pax7+/MyoD-) *in vitro* ⁶⁶. To investigate this, a recent study evaluated the role of Notch signaling on SC self-renewal by culturing canine satellite-cell-derived myoblasts on polystyrene culture plates coated with IgG-bound Notch ligand Delta1^{ext} ⁶³. Upon transplantation, the myoblasts that had been expanded on Notch ligand contributed to muscle regeneration as efficiently as freshly isolated myoblasts. Furthermore, the Delta1^{ext}-expanded cells generated stem cells *in vivo* – in other words, they were capable of self-renewal. This was shown by the engraftment of the Delta1^{ext}-expanded cells in secondary recipients ⁶³. These experimental outcomes show that Notch signaling is important to SC self-renewal, and that manipulation of Notch should be considered for *ex-vivo* expansion protocols.

In addition to Notch signaling, the Wnt pathway is known to contribute to SC self-renewal and cell-fate choice *in vivo* ⁸⁴⁻⁸⁶. Wnt7a, but not Wnt3a, was shown to activate planar cell division (see above), thereby promoting symmetric satellite-cell expansion *in vivo* ⁸⁵. It can be assumed that activation of the Wnt pathway helps to induce the self-renewing expansion of cultured SCs. Indeed, Wnt7a was shown to promote self-renewing division of Pax7+/MyoD- SCs, but only in isolated myofiber cultures and not in primary myoblasts grown on a regular culture dish ⁸⁵. Le Grand and colleagues determined that stimulation of self-renewing division by Wnt7a proceeded through the Wnt planar polarity pathway (PCP) ⁸⁵. This indicated that maintenance of cell polarity is essential to mediating the effect of Wnt7a. In myofiber cultures, SCs are in their natural environment and cell polarity is maintained ⁶⁶, while in regular 2D cultures polarity is lost. Although the study of Le Grand and colleagues showed that Wnt activity regulated symmetric self-renewing expansion of SCs, pharmacological stimulation of Wnt activity may not be sufficient. Instead it may be necessary to reconstruct the niche *in vitro*. For instance, to maximize benefit from soluble factors (such as Wnt7a) that promote self-renewing divisions of cultured SCs, it may be necessary to optimize the culture substrate (discussed below).

Expanding SCs under hypoxic conditions

Tissue stem-cell niches, including those housing SCs ⁸⁷, tend to be hypoxic, a condition that may be important for the function and survival of stem cells. In line with this, quiescent SC survived and retained regenerative activity in postmortem muscle tissue and severe hypoxia was found to be essential for the maintenance of these highly regenerative cells ⁸⁸.

Based on these and other observations, it has been suggested that culturing stem cells in hypoxic conditions may more closely approach the *in-vivo* situation and promote their stem-cell function. This was initially shown for neural crest ⁸⁹ and CNS ⁹⁰ stem cells. Hypoxia was also found to increase the efficiency of generating iPS cells ⁹¹. In addition, the differentiation of mouse myogenic cells grown under hypoxia was inhibited ^{92,93}, presumably through increased degradation of MyoD ⁹². The effect of hypoxia, which was shown to depend on Notch activity, activated Notch downstream genes through binding of the Notch intracel-

lular domain with HIF1 α ⁹³. In line with this finding and the effect of Notch activity on the self-renewing expansion of cultured myogenic cells ⁶³, hypoxia was found to increase the self-renewing cell divisions of mouse SCs and to enhance their engraftment potential ⁹⁴. Interestingly, hypoxia was also shown to induce myogenic proliferation of human muscle progenitors ⁹⁵, but as the effect on engraftment potential has not yet been determined, it remains to be determined whether the cells underwent self-renewal divisions.

An *in-vivo* tissue chamber model has been used to demonstrate that engraftment efficiency is increased by exposing (rat) muscle cells to hypoxic conditions before transplantation, a procedure called preconditioning (see below) ⁹⁶. The beneficial effect is thought to reside in the cells' adjustment to the hypoxic environment of the host muscle. Taken together, the increased regenerative potential of cells expanded in hypoxic conditions may be multifactorial, but offers a minimally invasive approach to improving the regenerative potential of stem-cell cultures.

Maintaining stem cells in suspension/spheroid culture

When cultured under low adhesion conditions, cells isolated from different tissues, including the breast ⁹⁷, heart ⁹⁸ and endothelium ⁹⁹, spontaneously aggregate and form spheres. While differentiated cells stop dividing under these conditions, stem cells continue to proliferate ¹⁰⁰, providing a relatively simple approach to enrich for tissue-specific stem cells. The effect of sphere-culture may be explained by their different cellular organization, which is closer to that *in vivo*, but also by altered biophysical signals resulting from a change in cell morphology and loss of contact with the substrate. Irrespective of the mechanism, the stem-cell properties of both mouse and human muscle stem cells appear to be preserved in spheroid culture and to result in enrichment of engraftable cells during expansion under these conditions ¹⁰¹⁻¹⁰³. Interestingly, human muscle cells could be expanded for at least 5 months under spheroid conditions and could undergo 40 population doublings before going into senescence ¹⁰². While this strategy may yield sufficient number of cells for treatment, it should be noted that the study using human muscle cells did not determine the engraftment efficiency of the myosphere cultures ¹⁰².

Inducing SC activation and proliferation: a two-step approach

After activation, *in-vivo* quiescent SCs enter the cell cycle and proliferate ¹⁰⁴. Most of the population progresses to committed myoblasts, which continue to divide for limited a number of cycles before differentiating into myocytes. The activation of SCs is dependent on several factors including sphingolipid signaling ¹⁰⁵, NO production (which results *in vivo* in release of HGF from the ECM) ¹⁰⁶; and growth factors (bFGF, IGF, IL-6). Several studies indicate that these signals also promote SC proliferation *in vitro* and may be used to rapidly expand the isolated muscle cells. As discussed above, expanding SC-derived cultures under proliferation conditions dramatically reduces their regenerative potential ⁷. However, this

strategy may currently be the only option for expanding human muscle progenitors. Unlike murine cultures, human muscle progenitor cells do not proliferate extensively *in vitro*, and undergo a limited number of divisions before entering senescence¹⁰⁷. Unfortunately, not much is known on the specific factors that promote the proliferative capacity of human cells. Some pathways, including IGF-signaling¹⁰⁸ and the TGF-beta pathway¹⁰⁹ (myostatin, a member of the TGF-beta superfamily, negatively affects muscle progenitor proliferation), control the proliferative activity of human muscle progenitors. The maintenance of the proliferative potential of human muscle progenitors is important not only for their eventual clinical applications, but even more to facilitate the study of the behavior of these cells in culture.

Once the conditions for efficiently expanding human muscle progenitors have been established, strategies should be followed to restore or increase the regenerative potential just prior to transplantation. This suggests that a two-step approach should be developed to obtain human muscle-regenerative cells as depicted in Figure 2 (indicated by the red arrows). Several approaches have been described that can be used to achieve this, including preconditioning, exposing the cells to hypoxia, or limiting oxidative stress in the transplanted population.

The first of these approaches, preconditioning, is defined as the exposure to a sublethal insult prior to transplantation in order to induce a protective response before transplantation that will allow the cells to better survive the hostile environment of the host tissue. Preconditioning has been studied mainly in the context of whole-organ transplantations, but recent studies suggest that cell-therapy strategies may also benefit from this procedure. In a tissue-engineering chamber model, preconditioning of myoblasts with the nitric oxide (NO) donor DETA-NONOate increased survival (and proliferation) after implantation⁹⁶.

With regard to hypoxia, we have stated above that preconditioning cells under hypoxic conditions to mimic the oxygen pressure in the host tissue was shown to enhance the transplantation efficiency of satellite cell-derived myoblasts⁹⁴. The beneficial effect of hypoxia was reported to increase engraftment almost 2-fold, but needs to be refined.

The third approach, increasing resistance to oxidative stress, may boost the engraftment potential of the cells expanded *ex vivo*. The damaged or dystrophic host muscle may prove to be a rather hostile environment for transplanted cells, being characterized by necrotic and apoptotic tissue, infiltration of inflammatory cells, and deposits of non-myogenic material. The identification of signals that adversely affect engraftment are as relevant as signals promoting engraftment. The transplanted cells may initially undergo increased levels of oxidative stress, which is thought to reduce the success of engraftment⁷⁷. It has been suggested that engraftment may be positively affected by adapting the conditions to limit the levels of oxidative stress in culture. Cells can be exposed to anti-oxidants, such as N-acetylcysteine or sodium ascorbate^{77,110}, during *ex-vivo* expansion or just before transplantation. Relative to engraftment potential of untreated cells, the transplantation of antioxidant-treated cells increased the formation of donor-host fibers about 1.7 fold⁷⁷.

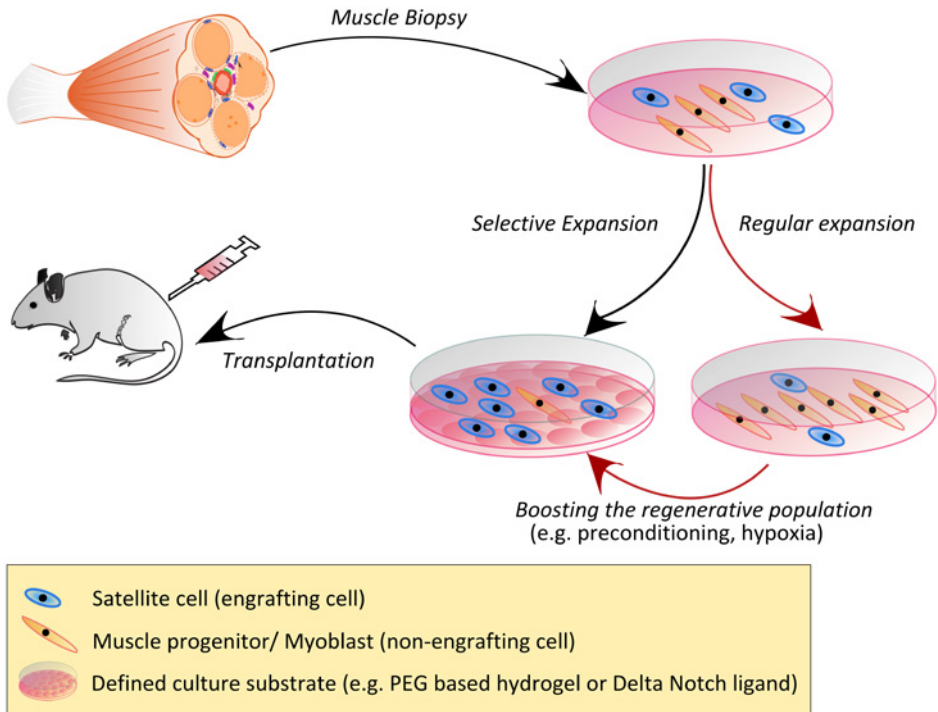


Figure 2: Selective expansion of muscle regenerative cells.

The figure depicts two different strategies to obtain cultures enriched with muscle regenerative cells: a ‘two-step’ approach and the selective expansion of highly regenerative cells. These procedures start with the establishment of a muscle culture from a small biopsy. The culture is heterogeneous and contains a subpopulation of cells capable of engraftment. **Two-step approach:** The cultures may be cultured under ‘regular’ conditions in order to expand the cells rapidly and extensively. Subsequently, strategies, such as preconditioning or exposing the cultures to hypoxic conditions (see text for details), can be applied to boost the regenerative potential of the expanded cells prior to transplantation (referred to as a two-step approach in the text; red arrows). The efficiency of this strategy may be low, because of the extensive loss of regenerative potential in the first step and the modest restoration (~2-fold) of the regenerative potential in the second step of the protocol. **Selective expansion:** Alternatively, the mechanisms of stem cell self-renewal *in vivo* may be applied to cultured cells (e.g. use of PEG-based hydrogel-based culture substrates or use of immobilized Notch ligand; see text). We hypothesize that such well-defined culture conditions promote the selective (self-renewing) expansion and, consequently, enrichment of engraftment-competent cells. It is expected that limited numbers of cells from such enriched cultures are required for efficient engraftment and regeneration.

Effect of stiffness of the culture substrate

The importance of defining the appropriate biophysical properties on the myogenic and regenerative potential of muscle cells has been shown in studies using various types of culture substrates to modify elastic stiffness. C2C12 myoblasts cultured on collagen-coated polyacrylamide gels, which approached the elasticity of skeletal muscle (~12 KPa), differentiated more efficiently than cells maintained on ‘hard’ plastic ¹¹¹. In addition, a direct correlation has been observed between the stiffness and proliferation rates (higher stiffness leads to increased proliferation ¹¹¹).

More recently, freshly isolated SCs cultured on polyethylene glycol (PEG)-based hydrogels with the same rigidity of muscle *in vivo* (~12 KPa) self-renewed *in vitro*, and contributed more efficiently to muscle regeneration *in vivo* than SCs cultured on regular plastic substrates. In both studies, the use of both softer and harder substrates resulted in decreased performance in the *in-vitro* and *in-vivo* assays used, indicating there is an optimal culture substrate formulation. As suggested, it will be interesting to determine whether the number of regenerative cells may be further increased by combining elastic substrates with chemically-defined media.

120 Generation of muscle progenitor cells by reprogramming somatic cells

In recent years it has become possible to use transient expression of 3-4 transcription factors to reprogram somatic cells to induced pluripotent stem (iPS) cells^{112,113}. Phenotypically and functionally, iPS cells resemble embryonic stem (ES) cells, and can be expanded *in vitro* for many passages while maintaining both pluripotency and the ability to differentiate into cells of all three germ layers¹¹³. This also eliminates the ethical considerations associated with ES cells. On the basis of these properties, iPS cells can be proposed as an attractive alternative to somatic cells.

The clinical application of iPS technology faces two major challenges: 1) how these cells can be derived without altering the genome, and 2) how they can be differentiated to homogeneity of the desired cell type. Common methods of generating iPS cells use retroviral or lentiviral gene delivery with the risk of insertional mutagenesis. Proper differentiation is important not only to obtaining the cell type of choice, but also to eliminating remaining pluripotent cells, which can form teratomas when placed in the wrong (non-embryonic) environment. Recently, important progress has been made. Various methods for non-viral gene expression have been reported, including those using Cre recombinase-mediated transgene excision¹¹⁴ and gene expression via the non-integrating Sendai virus¹¹⁵.

A number of reports document the successful generation of myogenic progenitors from mouse and human iPS cells, and engraftment of these cells in mouse models for human muscular dystrophies¹¹⁶⁻¹²¹. Major differences between various studies include the protocol used for generating myogenic progenitors, the efficiencies of these efforts, and the capacities of the cells generated for showing long-term engraftment and functional improvement.

An efficient method that results in successful long-term engraftment and functional improvement (i.e. 8 months in the mouse) was reported recently by Darabi et al., who, on the basis of previous observations using mouse embryonic stem (ES) or iPS cells^{122,123}, used inducible expression of pax7 during embryo body formation of human iPS cells. A straightforward FACS sorting approach based on co-expressed GFP proved sufficient to purify myogenic progenitors to homogeneity; no teratomas were observed after transplantation. The endogenous markers used for purification in the mouse were PDGF +/-

Flk-^{122,123}, though it is unclear whether these markers may be used in human as well. Importantly, intramuscular injection into the Tibialis Anterior muscle of a mouse model for Duchenne Muscular Dystrophy resulted in successful engraftment and the partial restoration of dystrophin expression. Donor dystrophin expression was still present 46 weeks after transplantation. Similarly, muscle function improved and a fraction of engrafted cells contributed to the endogenous SC population, suggesting that the iPS-derived progenitors self-renewed *in vivo*.

This work thus presents an important proof of principle for using iPS cells in the long-term treatment of muscular dystrophy. Challenges for the future include: efficient 1) generation of human iPS cells, 2) gene correction, 3) cell differentiation without functionally changing the human genome; and 4) the efficient delivery of cells to various muscles using intravenous or intra-arterial administration.

CONCLUSION

Despite its promise and potential, cell-based therapy for muscular dystrophies is still in its infancy. Although the clinical efficacy of myoblasts has turned out to be rather disappointing, the identification of additional cell types or populations – especially satellite cells and vessel-associated cells that can regenerate muscle – provide new hope for cell-oriented therapy. Their specific properties would indicate use in the treatment of distinct muscular diseases, which require either systemic (vessel associated cells) or local delivery (SCs). The progress in the field of cell-based therapy for skeletal muscle is underscored by the stage I clinical trials with vessel-associated cells for the treatment of DMD that started in 2011, whose results are awaited impatiently. SCs have not advanced to this stage as of yet and several issues require attention.

Most of the work on SCs has been performed with murine cells, and it must still be determined whether the findings described above can be applied to human SCs. Although it has been established that mouse and human SCs share many properties (reviewed in¹²⁴) – including the ability to regenerate muscle upon transplantation – there are some striking differences. The isolation of human SCs is complicated by the lack of highly specific markers, and, despite some strong initial indications these cells, too, are *bona fide* stem cells, it remains uncertain whether they self-renew *in vivo*¹²⁵.

The progress with human muscle progenitors is dependent on methods to overcome their limited proliferative potential in culture. In the short term, the ‘two-step’ approach discussed above (Figure 2) may be the most feasible strategy for human muscle progenitors, but strategies such as preconditioning and oxidative stress increase regenerative potential only modestly. The expansion of self-renewing cells seen in murine cultures would greatly increase the regenerative potential of the cultures that will be used for transplantation.

In this respect, the identification of the reserve cell (RC) in human muscle progenitor cultures ¹²⁶ is very promising. Like their counterparts in mice ^{51,127}, human muscle progenitor cultures have been shown to harbor a population of reserve cells (RC). Reserve cells are mononuclear cells that, under differentiation conditions, escape from differentiation and are thought to have properties of muscle stem cells ^{126,127}. It would be of major future interest and clinical importance to identify the mechanisms or factors that contribute to their specific maintenance or expansion *ex vivo*.

The next major milestone that can be envisioned for human muscle progenitors would be the evaluation of their therapeutic potential in a relevant (pre-) clinical setting that involves the isolation of human SCs, their expansion and finally transplantation to a suitable animal model. Only under these conditions the putative therapeutic potential of expanded human SC-like muscle progenitors can be evaluated. The importance of the immune system and its avoidance to engraftment success dictates that an animal model should be used that develops a relevant (i.e. human) immune response against the transplanted cells. The animal model should also make it possible to quantify the change in muscle function after cell transplantation. Given these requirements, it will be valuable to develop a humanized mouse model ¹²⁸ with a muscle phenotype. Such a model will also be valuable to the various laboratories that aim to use human muscle-regenerative cells for therapy.

A general issue of importance associated with cell-therapy is safety. The transplantation of C2C12 myoblasts, a myoblast cell line established in the late 1970s by Yaffe and Saxel ¹²⁹ was associated with muscle regeneration, but also with a propensity for generating tumors under certain conditions *in vivo*. It is thought that the cells may acquire a certain level of genomic instability and subsequent tumorigenic activity during the extended *in-vitro* expansion. Indeed, it has recently been shown that MDSCs acquired a transformational phenotype when expanded over 200 population doublings *ex vivo* ¹³⁰. This further underscores the importance of defining optimized conditions for expanding cells with the highest regenerative potential that may already achieve a functional effect at reduced numbers and require minimal expansion *ex vivo*.

A major advantage of including an *ex-vivo* expansion phase is that quality-control parameters can be implemented and Good Manufacturing Practice (GMP) guidelines be applied (see (<http://www.emea.europa.eu/>), enabling the generation of highly reproducible cell-products. More than any other technology, iPS offers the potential to generate large batches of well-defined regenerative cells that can be stored until use.

In conclusion, muscle regenerative cells remain attractive novel tools for the treatment of muscle disorders and much progress in understanding the behavior of these cells *in vitro* and *in vivo* has been made. However, it is also clear that several challenges, both with respect to practical issues and regulations, remain before introduction of a cell-based therapy for the treatment of muscle disorders becomes reality.

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Stem cell based gene therapy

for Pompe's disease

acid alpha-glucosidase

Integrations

Hematopoietic stem cells

Muscle

Mouse

Single intervention

Lentivirus

Future

Storage

ERT

Transplantation

Costs

Glycogen

Immune tolerance

Transplantation

DISCUSSION PART 1

GENERAL DISCUSSION

In this thesis, the development of lentiviral mediated hematopoietic stem cell (HSC) gene therapy for Pompe's disease is discussed.

This therapy is based on hematopoietic stem cell transplantation in an autologous setting. The patients' own cells will be modified with integrating lentiviral vectors that insert a functional *GAA* gene into the HSCs. The HSCs have the ability to self-renew; the inserted gene is not lost during the many cell divisions over time. Also, the gene is expressed throughout the whole hematopoietic lineage; *GAA* is able to reach the target tissues in the body by the bloodstream and cross-correction can occur.

Ideally, this therapy provides with a single intervention a permanent treatment for Pompe's disease, with minimal risks of transplant related morbidity and mortality, no immune responses against the enzyme, and a minimal risk of vector-related oncogenesis.

The *Gaa*^{-/-} mouse model is used in the studies described in this thesis. The *Gaa*^{-/-} mouse model is a good representative of Pompe's disease in humans, as it shows a progressive storage of glycogen with concomitant loss of muscle function; profound cardio-myopathy, respiratory insufficiency and skeletal muscle function weakness.

In Chapter 2 we demonstrate the promise of gene therapy for Pompe's disease as a proof-of-principle. We have used the native *GAA* transgene driven by the SFFV promoter to obtain above normal *GAA* concentrations in most tissues evaluated, which led to a reduction of glycogen in those tissues. Most importantly, we were able to restore cardiac function. This finding is of utmost importance, since patients with the most severe type of Pompe's disease, the classic-infantile patients, die within the first year of life because of cardiac and/or respiratory failure. We have observed a positive improvement of respiratory functions in mice treated with genetically modified HSCs, but the treated mice did not perform as the healthy control mice. Higher enzyme levels in respiratory muscles might improve respiratory function. One other feature we examined is skeletal muscle. This is the main tissue affected in Pompe's disease and can vary from a complete myopathy to a minor weakness in proximal muscles. We have addressed the skeletal muscle function in three ways. First, grip strength; to determine the strength of the front and/or hind limbs of the mice. Second, we measured the latency on the rotarod; an accelerating rod that combines motor function and muscle function. Finally, we observed the time the mice ran in voluntary running wheels. Since it is voluntary, it combines muscle function, motor function and fatigue. In all the muscle tests, we observed a large and clinically relevant

improvement of the gene therapy treated *Gaa*^{-/-} mice. But none of them performed as well as healthy mice.

One of the major challenges in ERT in Pompe patients is the formation of antibodies against the GAA; this will be later discussed in more detail. We demonstrate that by the use of genetically modified hematopoietic stem cells, immune tolerance for GAA is be obtained.

In Chapter 3 we describe experiments with an improved sequence of the *GAA* gene to evaluate the possibility of complete clearance of glycogen and restoration of skeletal muscle function. Codon-optimization of the transgene has been extensively used in other studies as well and showed a large improvement in transgene expression in these studies¹⁻³. GenScript (GenScript USA Inc.) performed the codon-optimization; they designed an algorithm that optimizes the gene sequences while taking in consideration different parameters that are crucial to transcription, translation and folding of the protein⁴. Following the same gene therapy strategy as in Chapter 2, we obtained a much higher expression of *GAA* in all tissues evaluated when compared to the native sequence in Chapter 2, including skeletal muscle and brain. A profound storage of glycogen is observed in the brain of *Gaa*^{-/-} mice, whereas the gene therapy treated mice show a reduction of glycogen in the brain. We demonstrate that complete glycogen clearance is possible, if enough GAA is present to degrade and prevent glycogen accumulation. These results with the optimized transgene exceeds the outcome with ERT on skeletal muscle and brain.

To address safety, integration patterns and sites of integration of the lentiviral vector were addressed. So far we have not observed any adverse effects related to lentiviral vector integration. The lentiviral vector does not show a higher preference for proto-oncogenes than other lentiviral vectors or in silico calculation.

Chapter 4 is a short original research communication about the importance of timing of the gene therapy treatment and the use of one transgene copy per cell. We have transplanted *Gaa*^{-/-} mice at different ages to evaluate the effect of treatment in different stages of disease progression. Ideally, one integration per cell would be preferred in a clinical setting; therefore we assessed the effect of one copy per cell in combination with transplantation at different ages. A reduction of glycogen in heart and liver was obtained, even when the mice were treated at one year of age. However, we did not observe a significant reduction of glycogen in skeletal muscle. It might be that one lentiviral copy per cell is not enough to obtain sufficiently high GAA levels to reduce or prevent glycogen storage, but further testing is advised. The mice that we transplanted at a very young age (3 weeks) also had profound glycogen storage when we examined them 6 months later. Thus, we concluded that one lentiviral copy number per cell might be sufficient to prevent lysosomal glycogen storage when treated at very young age, even before 3 weeks, when the animals do not

yet have abundant glycogen storage, but is too little to revert the muscle pathology at advanced disease stage.

Chapter 5 is a review about the possibilities of stem cells obtained from skeletal muscle (like satellite cells) in the treatment of muscular disorders and includes Pompe's disease as an example. The use of muscle stem cells for Pompe's disease could be useful as an additional therapy to ERT or gene therapy. For example in case the skeletal muscle has been damaged to quite some extent that additional repair by satellite cell proliferation and differentiation might be required besides ERT or gene therapy.

Regenerative potential of skeletal muscle of *Gaa*^{-/-} mice

In the classic-infantile form of Pompe's disease, the skeletal muscle is severely damaged. To investigate whether this damage is reversible, we studied the regeneration potential of muscle in *GAA*^{-/-} mice. Satellite cells are essential for the regeneration of muscle fibers, and the finding of centralized nuclei marks the regeneration of a particular muscle fiber. We have counted the number of regenerating muscle fibers in 5 months old mice (Table 1) and found that their number in *Gaa*^{-/-} mice exceeds their number in healthy mice. This implies that there is a process of constant regeneration ongoing in these mice.

Further investigations with respect to the regenerative potential of the satellite cells in Pompe's disease have not been possible within the time frame of the project. However, so far it is clear that satellite cells and muscle repair processes play a role in the pathophysiology of Pompe's disease. They can be a potential target in finding the optimal therapy for Pompe's disease, and could lead to a combination of different types of intervention.

Table 1: Regenerative potential of *Gaa*^{-/-} mice. QF: quadriceps femoris, TA: tibialis anterior, Wt: wild-type animals.

	Regenerating fibers QF (%)	Regenerating fibers TA (%)
<i>Gaa</i> ^{-/-}	24,2	15,2
Wt	2,1	1,0

Exercise has a positive effect on muscle endurance in a mouse model for Pompe's disease

Recent studies have shown the benefit patients with Pompe's disease can have when exercising on a regular basis^{5,6}. The combination of a constant supply of GAA and an exercise protocol can be in some patients of therapeutic value, most likely due to strengthening of the skeletal muscles. In one study, the effect of nutrition and exercise on the progression of the disease has been observed⁷. The authors showed that training and a low-carbohydrate and high-protein diet can slow the deterioration of muscle function and prolong life.

To evaluate the effect of exercise in our Pompe's disease mouse model, we observed the distance the mice ran voluntarily in running wheels. The setup consisted of cages equipped with a running wheel and a computer system that register the time, speed and distance mice run. Mice were 6-8 weeks old at the start of the experiment, which lasted for one year. With intervals of about 30 days, the mice were allowed to use the running wheel continuously for 14 days on average, after which they were returned to their home cage. Surprisingly, we did not observe any difference in daily running wheel activity between wild type and *Gaa*^{-/-} mice (Figure 1, depicted are the first 7 days and mice were 6 months of age).

When we measured the distance that age-matched *Gaa*^{-/-} mice naïve to the wheels (KO untrained 6 months) ran, there was a statistically significant difference between the performance of the trained and the naïve *Gaa*^{-/-} mice: naïve *Gaa*^{-/-} mice performed worse than trained *Gaa*^{-/-} mice (Figure 1). Moreover, 12-month-old naïve *Gaa*^{-/-} mice performed worse than 6-month-old *Gaa*^{-/-} naïve mice, confirming the deterioration of muscle function during the progression of the disease. Our findings corroborate the observations made in human patients that voluntary exercise benefits the course of the disease.

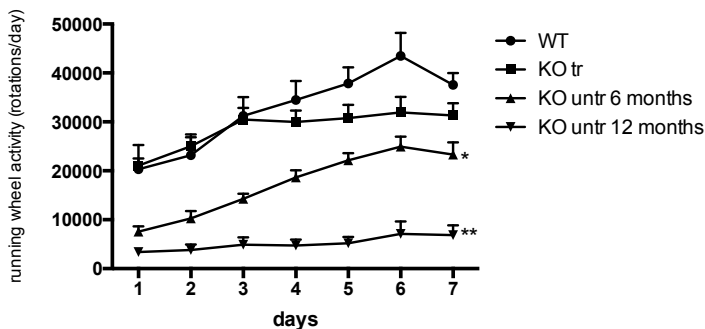


Figure 1: Daily voluntarily running wheel activity in Pompe's disease mice.

The mice were evaluated on their running capacities at 6 months of age in this figure. WT; wild type, KO tr: *Gaa*^{-/-} in training, KO untr: naïve *Gaa*^{-/-} mice. * $p < 0.05$, ** $p < 0.005$ vs wt.

At regular intervals, immediately before the training periods, we have evaluated the effect of training on muscle performance using grip strength and rotarod performance tests (Figure 2). We observed significant differences in grip strength and rotarod performance between wild type and *Gaa*^{-/-} mice in training: from 2 months of age onwards, the grip strength was lower for *Gaa*^{-/-} compared to wild type mice and from 4 months of age onwards, a significant decrease of rotarod performance was observed in *Gaa*^{-/-} mice. Because we observed no differences in running wheel activity between both groups of mice, these findings suggest that training does not have a direct effect on muscle strength, but rather on muscle endurance.

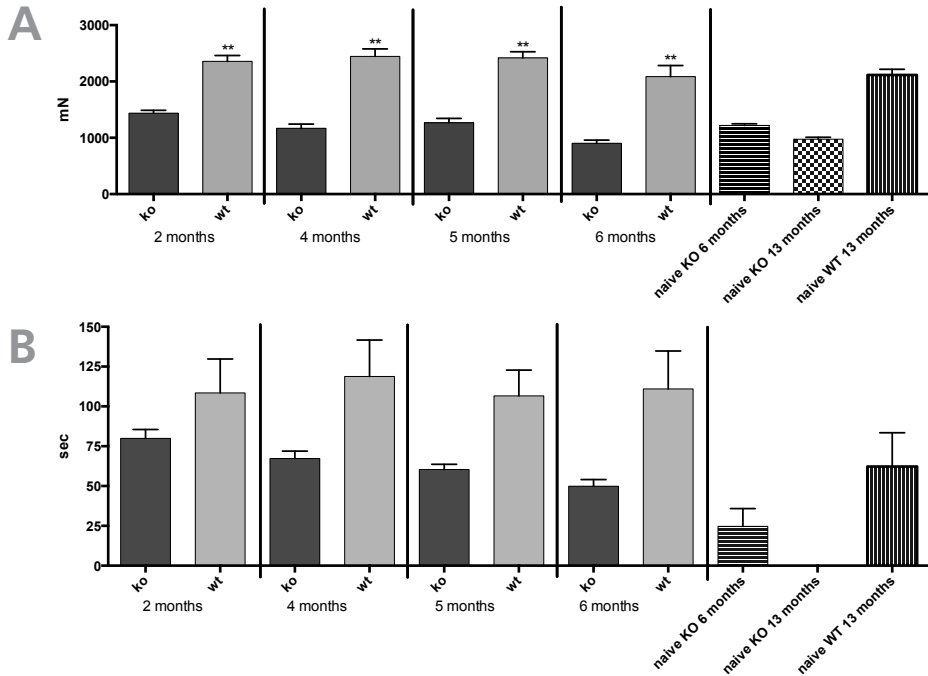


Figure 2: Grip Strength and rotarod performance.

The mice were evaluated before the training periods on their strength in all 4 paws (A, significant difference with $p < 0.0001$ at 4, 5 and 6 months of age) and accelerating rotarod performance (B, significant difference between ko and wt at 4, 5 and 6 months of age ($p < 0.05$ at 4 months, $p < 0.01$ at 5 and 6 months)).

At 6 months of age, behavior of these animals was tested in an open field for 10 minutes. We analyzed the time the mice were moving, in how many episodes they moved and the total distance they moved (Figure 3). Overall, the *Gaa*^{-/-} mice that had been in training performed better than their age-matched naïve controls.

We emphasize that the mice were not on a forced exercise scheme and that no alterations were made in their nutrition. During the time of the project, we were not able to further investigate the effect of training in gene therapy treated mice. To obtain a better understanding of the difference in the various muscles groups affected and how to train the mice as effectively as possible to obtain a therapeutic benefit in addition to ERT, more experiments are required. Promoting training could have a positive impact on the treatment regimen of Pompe's disease patients. We hypothesize that if exercise can contribute to a positive effect on glycogen clearance in skeletal muscle(s), ideally less integrations of the viral vector would be needed to obtain therapeutic benefit.

Neurological involvement in Pompe's disease

The brain displays a broad cellular heterogeneity⁸. Glycogen is stored throughout the entire cell population, but predominantly in the peripheral astrocytic processes. Astrocytes

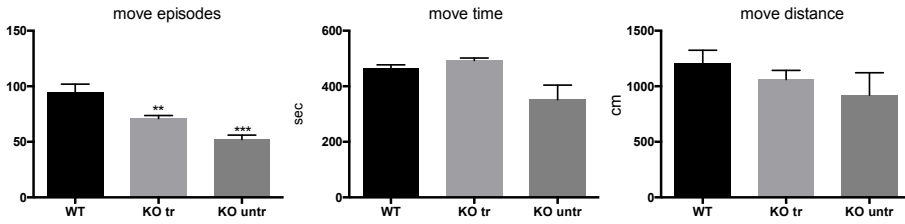


Figure 3: Open field tests.

The behavior of the animals was tested in an open field at 6 months of age. WT; wild type, KO tr: *Gaa*^{-/-} in training, KO untr: naïve *Gaa*^{-/-} mice. ** $p < 0.005$, *** $p < 0.001$ vs wt

surround the neurons, so when the demand for glucose is high, there is a fast supply of energy at hand⁹. In the cerebellum specialized astrocytes (Bergmann glia) form complex interactions with the Purkinje cells^{10,11}. In Pompe's disease, few autopsies have been done on deceased patients^{12,13} and have demonstrated glycogen accumulation in glia cells, Purkinje cells, cortical neurons and motor neurons¹⁴⁻¹⁷. The patients do not display noticeable neurological deficits, despite of the glycogen storage in the central nervous system (CNS) and peripheral nervous system (PNS). In our mouse model we observed a high concentration of glycogen within the brain as well. Despite this massive storage of glycogen, no obvious neurological involvement is seen in the *Gaa*^{-/-} mice so far.

In Chapter 3 we show that a slight elevation of GAA in the brain can lead to a reduction of glycogen, up to normal levels. We hypothesize that the observed correction of the glycogen storage in the brain is effectuated by the blood derived, *GAA* gene-corrected macrophages that have crossed the blood brain barrier.

Immune cells within the CNS are resident microglia and monocyte-derived macrophages. Microglia are considered macrophages and these cells are capable of phagocytosis to protect the neurons in the CNS by cleaning up plaques, damaged neurons, infections, etcetera¹⁸. The monocyte-derived macrophages can infiltrate the CNS in case of neurological damage (Figure 4)^{19,20}. For example in neurodegenerative diseases or in case of conditional ablation, the monocyte-derived macrophages are recruited from the blood by T-helper lymphocytes to the CNS^{21,22}. At the moment, it is not possible to distinguish the two cell populations (resident microglia and monocyte-derived macrophages) from each other by immunohistochemical techniques. In Chapter 3 we describe that some of the F4/80 (a marker for microglia) positive cells in the brain are also *GAA*-positive and some are not. We hypothesize that the *GAA*-positive F4/80 cells (monocyte-derived macrophages) are the cells that express *GAA* in the brain and deliver the enzyme by cross-correction to the other cells in the brain like the neurons and astrocytes²³.

Safety issues

The ability of integrating vectors to insert a gene into the genome can be of concern when the integration takes place near a proto-oncogene and activates the proto-oncogene or

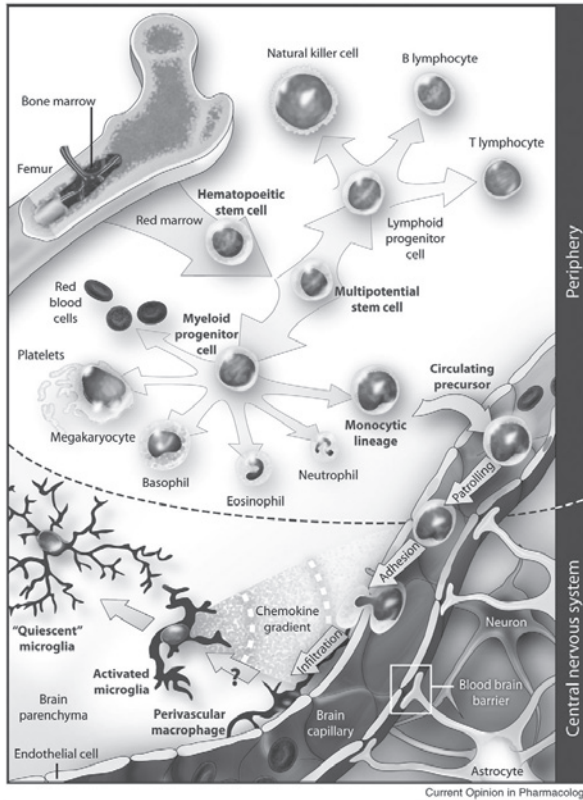


Figure 4: Bone marrow derived microglia migrate from the bone marrow to the brain (reused with permission of publisher)²⁰.

near a tumor suppressor gene and inactivates the tumor suppressor gene. Although the integration pattern of lentiviral vectors is considered as random, the more copies per cell, the higher the risk is of unwanted insertional mutagenesis. The major advantage of the codon-optimized sequence is that fewer copies per cell are needed to obtain therapeutic enzyme levels, thereby reducing the risk of insertional mutagenesis. Potentially, weaker promoters could replace the strong SFFV promoter used in these studies. However, by using weaker promoters, the number of integrations might need to go up in order to obtain the same therapeutic effect as obtained with a stronger promoter. So far, we have tested different promoters, like the PGK promoter (phosphoglycerate kinase), since this promoter has shown to provide therapeutic levels of enzyme in other diseases^{24,25} and is currently being tested in a clinical trial in a lentiviral vector to treat metachromatic leukodystrophy (MLD)^{25,26}.

Unfortunately, even in combination with the codon-optimized transgene, we were not able to obtain GAA levels as high as with the SFFV promoter, and no complete reduction of glycogen occurred, as can be seen in Figure 5.

In Pompe's disease high levels of the deficient enzyme are needed in order to clear the accumulated glycogen. This has been shown in the case of ERT and in our gene therapy strategy as shown in Chapter 3. In comparison, the dosing of Fabrazyme®, the recombinant enzyme prescribed to patients with Fabry's disease, another LSD in which ERT is the current treatment, is 20 times less than the dosing recommendation of Myozyme® for Pompe's disease^{27,28}. This is why we need to pursue development of HSC based gene therapy for Pompe's disease in a clinical setting using a strong promoter.

Gene therapy *versus* ERT

An inconvenience of ERT is that intravenous infusions of GAA need to be given every other week and sometimes even every week. With every infusion the patients receive a bolus of GAA of which the plasma half-life is only 2.3 hours²⁹. The intracellular half-life of the enzyme, once taken up by the tissues, is approximately 10 days²⁹. The major advantage of gene therapy compared to the strict and chronic infusion regimen in ERT, is it that it provides in principle an eternal source and constant level of GAA in the blood cells (as described in Chapter 2 and 3)³⁰.

New symptoms after introduction of ERT

Despite the profound accumulation of glycogen patients with Pompe's disease have in the CNS, they do not show overt clinical neurologic symptoms. When ERT became available to the patients in 2006, their survival increased and other symptoms related to the disease were detected (especially in the classic-infantile form), which were previously not

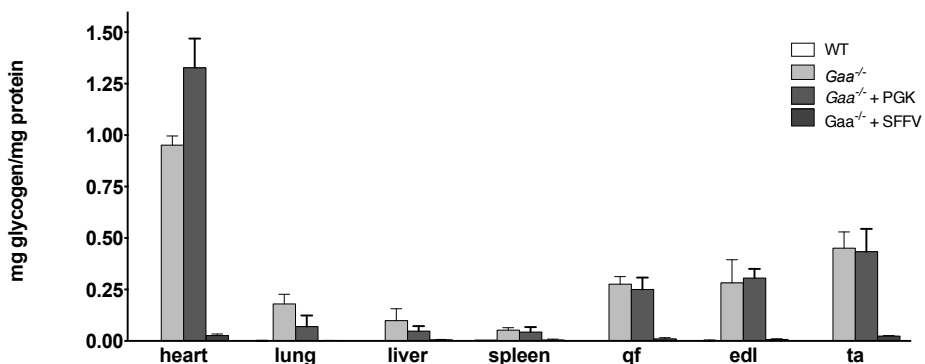


Figure 5: Comparison between SFFV and PGK promoter.

No significant improvement in glycogen reduction is measured. The mice were transplanted and sacrificed under the same conditions. WT: wild type: not visible on this graph. Glycogen values in WT are: Heart: 0.0004 mg glycogen/mg protein, Lung: 0.0007 mg glycogen/mg protein Liver: 0.0014 mg glycogen/mg protein Spleen: 0.004 mg glycogen/mg protein QF: 0.0006 mg glycogen/mg protein EDL: 0.0021 mg glycogen/mg protein TA: 0.0006 mg glycogen/mg protein. QF: quadriceps femoris, EDL: extensor digitorum longus, TA: tibialis anterior.

observed simply because the patients did not survive long enough to acquire these symptoms. Examples of symptoms introduced after the introduction of ERT are hearing loss and problems swallowing³¹⁻³³. This implicates that other symptoms related to the disease might be revealed as the survival of these patients increase following treatment and neurological symptoms might be one of them.

Current developments in gene therapy for Pompe's disease

The studies described in this thesis shine a new light on the development of gene therapy for Pompe's disease. Up to now, most of the research regarding gene therapy for Pompe's disease involves AAV vectors, but the use of these vectors has some limitations, especially for a systemic disease like Pompe's. The major limitation of AAV vectors is that they do not induce immune tolerance; barring the risk of immune reactions to the vector capsid or the transgene. An advantage of AAV vectors is the existence of different serotypes; the virus can transduce a wide variety of tissues and cells *in vivo*.

Byrne and colleagues^{34,35}, have demonstrated a new method to deliver the GAA gene to the diaphragm of Pompe patients using an AAV1 vector. In preclinical experiments they used a glycerin-based gel containing the vector and applied to the diaphragm of *Gaa*^{-/-} mice. It resulted in proper delivery of the GAA gene followed by clearance of the lysosomal glycogen to an extent that the ventilator function of the mice was partially restored. In a subsequent Phase I/II clinical trial in children with Pompe's disease (age 18-180 months) that were ventilator dependent, they assessed whether the administration of AAV1 with GAA can lead to acceptable safety outcome and functional changes in respiratory function³⁶. These patients were kept on ERT during the locally applied gene therapy and therefore it was difficult to conclude whether the improvements were due to the gene therapy, the ERT or a combination of both. The preliminary outcome of this trial indicated that certain ventilator parameters improved after gene therapy treatment, such as the tidal volume. Other parameters such as maximal inspiratory pressure did not improve. So far, a T cell-mediated response to the vector (or capsid) was not observed.

Localized delivery of genes can be an attractive approach in other diseases too, for example in Leber's hereditary optic neuropathy (LHON); a neurodegenerative disorder that causes blindness in young adults^{37,38}. However, the use of AAV based vectors for the treatment of systemic disorders is not seen as the ultimate solution because of the immunological response to the capsid and the inability to repeat the treatment.

Gene therapy and immune responses

Immune responses directed against the recombinant human GAA can occur in patients with Pompe's disease receiving ERT, and these can counteract the effects of treatment. HSC transplantation is known to induce tolerance by modifying the immune system. Among

the descendants of the HSC are immune cells that play an important role in the response to foreign material entering the body, such as viruses, bacteria and other biological pathogens. Some patients with classic-infantile Pompe's disease do not produce any GAA in any of their cells including the HSCs and the immune cells. Therefore, the immune system will consider the recombinant GAA administered in ERT as a foreign substance and will start to produce antibodies against it. However, in an autologous HSC transplantation setting with genetically modified HSCs, the immune cells themselves express the GAA, and are therefore less likely to consider the recombinant GAA as foreign substance. In Chapter 2 we show this in the murine model of Pompe's disease where we did not find an antibody response after injections of recombinant human GAA in mice that previously received HSC based gene therapy. A different and more challenging task is to induce immune tolerance while the patients already have been exposed to GAA and have developed antibodies against the GAA. Currently attempts are made to prevent and combat antibody formation with drugs³⁹. One of the current approaches to suppress an immune response to GAA is to use drugs like Rituximab. This is a monoclonal antibody against CD20 on B-cells and effectively depletes B cells⁴⁰. However, the use of drugs that constantly suppress the immune system is not an ideal option, since they compromise the immune system and the patients become more sensitive to infections^{41,42}.

A widely used strategy to circumvent immune responses is the use of microRNAs. MicroRNAs are 21-22 nucleotides long non-coding sequences that are (partially) complementary to the mRNA molecule. When a microRNA binds to its complementary sequence, translation or repression of the mRNA occurs. The microRNA sequence can be inserted in the lentiviral vector and upon expression will degrade or repress the mRNA in cells in which the microRNA is expressed to avoid immune responses to the transgene⁴³. In some LSDs, like Krabbe (globoid cell leukodystrophy, GLD), the expressed enzyme (galactosylceramidase, GALC) is toxic for the HSCs. In order to prevent the expression of GALC in the HSCs a microRNA (miRNA-126) is used. This microRNA prevents expression of the transgene in stem cells and direct progenitors but allows GALC expression in mature hematopoietic cells, which leads to therapeutic benefit in a murine model without toxicity⁴⁴.

In the case of Pompe's disease it is not crucial to use microRNAs when a patient has not developed an immune response to the GAA; since the patient will gain immune tolerance for GAA by the HSC transplantation. The use of miRNAs to prevent the expression of GAA in antigen presenting cells can be of interest for patients who already have developed an immune response to the GAA.

Challenges of integrating lentiviral vectors

Gene therapy has shown great clinical potential over the years, but has as yet not received wide application. Fortunately, methods to deliver the therapeutic gene more efficiently to

the target tissues have been developed and several products are in clinical trial. To develop the most successful strategy for a certain disease, several factors need to be considered. For instance the difference between a complex genetic disorder versus a monogenic disease, whereby the latter is more likely to be successfully treated by the current gene therapy methods. Other factors to consider in the development of the most optimal vector for a certain disease include: i) systemic versus local delivery; ii) what needs to be achieved; a temporary effect or a sustained effect?; iii) what gene expression level is desired?.

Lentiviral vectors harbor several characteristics that make them optimal tools for gene transfer in HSCs. They are able to transduce dividing and non-dividing cells, provide sustained long term expression through integration into the host genome, transduce many different cell types (or specific cell types, dependent on the envelope used in the vector design), they are replication deficient, they have a low genotoxicity and they are relatively easy to produce and dose. One of the main advantages of lentiviral vectors is the stable integration into the host genome. As mentioned in the introduction, this is also a matter of concern since they potentially could insert their genetic content near proto-oncogenes or tumor-suppressor genes and alter their function. However, the insertion of lentiviral vectors appears to be fairly random as opposed to retroviral vector insertions; which display a preference for integration near active sites within in the genome⁴⁵. Studies in a tumor prone mouse model have demonstrated the lack of genotoxicity of the SIN lentiviral vector, indicating that it was not the promoter, but the lentiviral vector design that contributed to genotoxicity in initial studies that revealed integration near proto-oncogenes⁴⁶⁻⁴⁹. In the experiments reported in this thesis (Chapter 3), we have not observed a higher preference for integration of the transgene near proto-oncogenes than other lentiviral vectors with a different promoter-transgene combination.

Another risk of integrating vectors is clonal dominance. Clonal dominance is the survival of one or more distinct clones from a polyclonal population, which can proliferate and develop malignant manifestations⁵⁰. When there is no malignant manifestation due to insertional mutagenesis per se, clonal dominance is not considered as a potential risk and it does not matter whether the transgene expression is sustained by a polyclonal population or by an expanded dominant clone^{51,52}. Factors that can contribute to clonal expansion are considered to be the vector (type and design), therapeutic transgene and the disease setting. In some diseases, expression of the transgene can lead to a selective advantage to the cells that express the transgene. In clinical trials conducted with lentiviral vectors polyclonal patterns of viral integration were detected^{53,54}. Only a small number of HSCs were actually genetically modified, but reconstitution of the immune system was observed⁵⁵.

Different promoters for different applications

The promoter choice contributes to the level of transgene expression and tissue specificity⁵⁶. Promoters can be designed to react to external stimuli, the so-called inducible promoters, or to be tissue specific or specific for certain developmental stages⁵⁷.

Tissue-specific promoters are used to direct or enhance transgene expression in a specific cell type. For example erythroid specific promoters (like ankyrin-1, α -spectrin and β -globin), or enhancers (for example GATA-1 autoregulatory element, α -globin HS40 and β -globin LCR) are used to only express the transgene in the erythroid lineage^{58,59}. Richard and colleagues obtained superior results by combining an erythroid promoter (ankyrin-1) with two enhancers (GATA-1/HS40) to treat a murine model with erythropoietic protoporphyria⁶⁰. Another example of the use of a tissue-specific promoter to achieve transgene expression in APCs, is to use a HLA-DR α promoter. For instance, Cui et al showed transgene expression exclusively in major histocompatibility complex class II (MHCII) expressing APCs⁶¹. Tissue-specific promoters are commonly used in terminally differentiated tissue like liver, CNS and muscle^{56,62}.

Inducible promoters are used in disease settings that do not require ubiquitous expression of the transgene. Applications of this type of promoters can involve; regulation of the transgene expression levels within the therapeutic range or modulation of the transgene expression in response to disease evolution. Most commonly used inducible vector systems are the tetracycline or doxycycline dependent promoters^{63,64}. Other mechanisms involve heat, light or acetaldehyde⁶⁵. The majority of the inducible promoters are developed for the treatment of cancer^{56,62}.

Ubiquitously expressed promoters are used when the transgene expression is required in all cell types, and no tissue specificity is needed, e.g. in Pompe's disease. The promoter that we used in the experiments described in this thesis is a strong viral promoter, the spleen focus forming viral promoter (SFFV), which is known for its high expression levels in the hematopoietic system.

The expression of an integrated transgene can be repressed by the surrounding chromatin. To increase the efficacy of the expression of the transgene, chromatin insulators can be used; these are DNA sequences that can shield the promoters and enhancers from silencing by the surrounding chromatin. Examples are the ubiquitously acting chromatin-opening element (UCOE)⁶⁶ and the chicken hypersensitive site-4⁶⁷.

How to improve gene therapy vectors?

Methods to improve the delivery and/or uptake of the transgene product can include the addition of certain sequences to promote trafficking of the enzyme to the lysosomes or facilitate its maturation. These are just two examples of what can be achieved with optimized vector design and can be tested in a lentiviral setting. It goes beyond the scope of this

thesis to discuss all the possible adjustments to optimize a lentiviral vector, only two will be discussed below that are currently used in preclinical experiments for Pompe's disease.

An important factor in the efficacy of ERT for Pompe's disease is related to the mannose 6-phosphate receptor. The mannose 6-phosphate receptor carries out several functions essential for normal cell function. About 10% of the total mannose 6-phosphate receptors are located on the surface of the cell⁶⁸. They mediate endocytosis and uptake of exogenous lysosomal hydrolases into the lysosomes. They also bind insulin-like growth factor II (IGF-II); a growth promoting hormone and facilitate activation of a serine protease involved in apoptosis induced by cytotoxic T-cells⁶⁹. Because of its involvement in cell growth and function, the mannose 6-phosphate receptor is believed to act as a tumor suppressor⁶⁹. In the treatment of lysosomal storage disorders, the mannose 6-phosphate receptor is crucial for its role in binding and internalization of the therapeutic enzymes. To improve the uptake of recombinant human enzymes currently used for ERT, LeBowitz and colleagues have replaced a short N-terminal part of glucuronidase, the enzyme deficient in MPSVII, the N-terminal part of human IGF-II. This resulted in a higher affinity of the hybrid enzyme for the mannose-6-phosphate receptor⁷⁰. In Pompe's disease, this has been tested as well. A glycosylation-independent lysosomal targeting (GILT) signal, that includes a portion of IGF-II, was added to the GAA sequence. This N-terminally modified recombinant human GAA cleared the accumulated glycogen in skeletal muscles much more efficient than the unmodified enzyme⁷¹, and is currently tested in a Phase III clinical trial (trial number: NCT01230801). Also the chemical addition of mannose 6-phosphate residues to the carbohydrate chains of recombinant human GAA leads to better uptake and clearance of lysosomal glycogen GAA⁷².

The next part of the discussion will discuss the regulatory road to marketing authorization for gene therapeutic medicinal products with the preclinical experiments for the development of a gene therapy product for Pompe's disease described in this thesis in mind.

DISCUSSION PART 2

THE REGULATORY ROAD TO MARKETING AUTHORIZATION FOR GENE THERAPEUTIC MEDICINAL PRODUCTS.

Many of the preclinical gene therapy studies conducted in animals have shown promising therapeutic benefits, but only few applications have actually reached a further state of development. There is a clear understanding that identification of possible hold-ups in

the process from preclinical work to an actually registered advanced therapeutic medicinal product (ATMP) is crucial for the patient to benefit from all the outstanding developments in the research field. Here, we will try to clarify the regulatory road one has to take to get an ATMP licensed in Europe and in particular in The Netherlands. A fictional case of a clinical trial for stem cell based lentiviral gene therapy for Pompe's disease will be used as an example.

Introduction

Many of the preclinical gene therapy studies conducted in animals have shown promising therapeutic benefit, but few of them have actually reached a further state of development. There is a clear understanding that identification of possible hold-ups in the process from preclinical work to a registered advanced therapeutic medicinal product (ATMP) is crucial for the patient to benefit from the developments in the research field. Here, we will clarify the regulatory road one has to take to get an ATMP licensed in Europe, and in particular, in the Netherlands.

The first approved gene therapy medicinal product (GTMP), Glybera® (Box 1), received marketing approval from the European Medicines Agency (EMA) early 2013 and many lessons can be taken from the process. In summary, the hurdles during the licensing process for Glybera® were⁷³:

- Licensing process was initiated by researchers in academia, who are often inexperienced in licensing;
- due to limited experience the regulators had with GTMPs, they assessed the GTMP as if it was a conventional drug and initially did not recognize the exceptional status. Based on the number of patients involved, it should have been initially designated as an extremely orphan drug;
- small start-up companies lack sufficient financial resources to fund the long process of licensing a GTMP, especially when unforeseeable queries and problems are encountered along the way and the process involves more time than anticipated;
- Presenting the data to the regulators in a convincing and efficient way was challenging;
- in the course of the clinical trials the researchers changed the doses of the GTMP;
- conflicting advice by two advisory groups, the Committee for Advanced Therapies (CAT) and the Committee for Medicinal Products for Human Use (CHMP) led, in part, to the researchers changing the endpoints the trials (pancreatitis or triglyceride levels as endpoint);
- there were changes in the production platform.

In addition, we concluded that regulators showed risk-avoiding behavior; they were 'scared of the unknown'. In the end, Glybera received approval from both the CAT and CHMP for

marketing authorization under exceptional circumstances. As this was the first GT product licensed in the Western world, it started a new era for gene therapy.

Box 1

Glybera®

The first approved GTMP is alipogene tiparovec, or Glybera®, for the treatment of lipoprotein lipase deficiency (LPLD). Deficient lipoprotein lipase leads to failure of hydrolysis of plasma triglycerides resulting in hypertriglyceridemia. Patients with LPLD suffer from fat deposits in the skin and retina, abdominal pains, diabetes and cardiovascular disease. The disease is characterized by frequent episodes of pancreatitis, which can be fatal. The strategy proposed by UniQure (previously AMT) involves intramuscular injections of AAV-1 to mediate gene transfer of a functional LPL gene.

Clinical studies showed that alipogene tiparovec significantly reduced the triglycerides levels in plasma after a single dose of AAV-1 up to 14 weeks after administration and reoccurrence of acute pancreatitis reduced as well. The European Commission approved marketing authorization of Glybera® on November 2nd, 2012, under exceptional circumstances^{73,74}.

ATMPs

Advanced therapeutic medicinal products (ATMPs) comprise three fields of medicines for the use in humans: somatic cell therapy (CTMPs), gene therapy (GTMPs), and tissue engineering products (TEPs, will not be discussed any further)⁷⁴. These are innovative medicinal products investigated for different diseases.

According to the European Medicines Agency (EMA, “a regulatory agency that is responsible for the protection and promotion of public and animal health, through the evaluation and supervision of medicines for human and veterinary use” per the website www.ema.europa.eu), a CTMP is defined as:

“A biological medicinal product which contains or consists of cells or tissues that have been subject to substantial manipulation so that biological characteristics, physiological functions or structural properties relevant for the intended clinical use have been altered, or of cells or tissues that are not intended to be used for the same essential function(s) in the recipient and the donor. And the product is presented as having properties for, or is used in or administered to human beings with a view of treating, preventing or diagnosing a disease through the pharmacological, immunological or metabolic action of its cells or tissues.”⁷⁴

Some autologous or allogeneic hematopoietic stem cell transplantations (HSCT) are not included in the AMTP class CTMPs. If they are used for a non-homologous purpose, they are considered CTMPs (for example the local injection of HSCs into the site of a myocardial

infarction). In general, CTMPs are used to prevent, diagnose, or treat a specific disease by their biological mode of action via pharmacological and metabolic ways. When considering the HSCT in case of HSC mediated gene therapy, although the HSCs are placed back in their natural niche, they are substantially manipulated *ex vivo*; resultantly altering the natural biological activity of the cells by inserting a genetic sequence. This means that this therapy meets the two characteristics of GTMP according to the European Medicines Agency (EMA):

“A medicinal product that contains an active substance which contains or consists of a recombinant nucleic acid used in or administered to human beings with a view to regulating, repairing, replacing, adding or deleting a genetic sequence. And its therapeutic, prophylactic or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence”⁷⁴. GTMPs do not include vaccines against infectious diseases.

Regulatory agencies

Regulation of approval for clinical trials

One of major challenges many preclinical gene therapy projects encounter once they are ready for clinical trial is the fact that each country within the EU has complemented the common ATMP-regulation with its own flavor.

In the Netherlands, regulations for clinical trials with ATMPs involve several organizations: the Minister of Health, Welfare and Sport (VWS) thereby delegating its task to the Medicines Evaluation Board (College ter Beoordeling van Geneesmiddelen (CBG)), the Central Committee on Research Involving Human Subjects (Centrale Commissie Mensgebonden Onderzoek (CCMO)), Minister of Infrastructure and Environment (IenM), and the Office for Genetically Modified Organisms (Bureau Genetisch Gemodificeerde Organismen (Bureau GGO)). The latter is responsible for processing the IenM permit requests with additional advice in such processing provided by the Netherlands Commission on Genetic Modification (Commissie Genetische Modificatie (COGEM)). All of these organizations need to be in favor of proceeding before a clinical trial can be initiated.

The legal frameworks used by the different organizations listed above are based on European Union legislation and regulation. Since this is a very complex field, the Gene Therapy Office was established in 2004⁷⁵. The Gene Therapy Office serves as a point of contact and plays a central role in the coordination of the procedures between the investigator and the different assessment organizations. The Gene Therapy Office receives the applications from the investigator, including reports, and will communicate the decisions made by the different assessment bodies to the investigator (Figure 6).

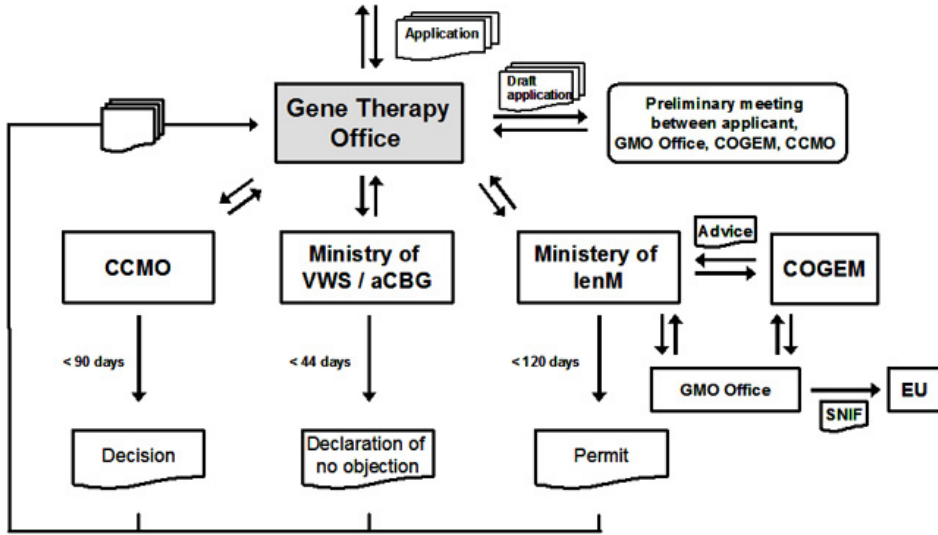


Figure 6: The role of the Gene Therapy Office, the mediator between investigator and assessment bodies in the Netherlands in clinical and preclinical studies⁷.

Besides a mediator for investigators and the official decision-making party regarding gene therapy applications, the Gene Therapy Office provides the sponsors, government, and investigators with information about gene therapy.

Regarding clinical trial approval, the Central Committee on Research Involving Human Subjects (CCMO, a medical ethical committee) is involved and will evaluate all clinical gene therapy research that involves human subjects. It assesses the proposal on the risks the proposed procedure holds, not only for the human subject, but also for society.

The Minister of Infrastructure and Environment (IenM) is responsible for the regulations that protect people and the environment during activities involving GTMPs, i.e. genetically modified organisms (GMOs). It is also responsible for policy development and regulations for GTMPs. The Netherlands Commission on Genetic Modification (COGEM) will advise the Minister of Infrastructure and Environment. It does not provide any advice on the risks for the human subjects, only on the risks for staff involved in the treatment, family members, and others. It will also inform on the social aspects and ethics around the proposed application of GTMPs and a possible introduction in the environment. The Office for Genetically Modified Organisms (Bureau GGO) is responsible for administrative procedures and technical implementation of the proposed GMO permit.

A declaration of no objection about the proposed GTMP should be given by the Minister of Health, Welfare and Sport, based on the act of Medicinal Research Involving Human Subjects. It has delegated this task to the Medicines Evaluation Board. They are responsible for the assessment, authorization of GTMPs, and monitor the safety of GTMPs within the Netherlands. They also share the responsibility for the marketing authorization of GTMPs in the European Union.

Regulations for marketing approval in EU

All ATMPs need to be evaluated and approved by a framework established by the European Union (EU) and the EMA (European Medicines Agency) before they can be marketed. Within the EMA, two regulatory bodies can be distinguished, the Committee for Advanced Therapies (CAT) and the Committee for Medicinal Products for Human Use (CHMP). The CAT prepares a draft proposal that will be discussed in the CHMP. After which, the CHMP will send its advice to the EU commission (Figure 7).

Requirements of GTMPs

Detailed guidelines of which studies are preferred regarding the quality, preclinical, and clinical aspects are available⁷⁶. In these guidelines, the EMA (and in particular the CAT) defines scientific principles and provides guidance in an effort to harmonize the approach of GTMP development in the EU. However, since every GTMP is a unique product that will be evaluated individually, the final requirements may vary by product.

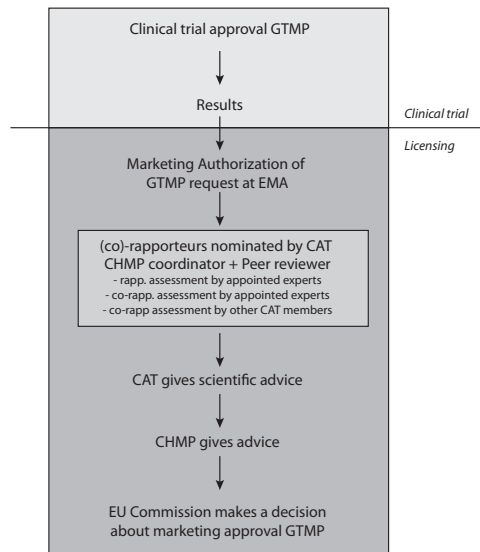


Figure 7: Roadmap from clinical trial to market authorization⁸.

The primary objective of the evaluation of pre-clinical data is to provide sufficient data to allow a risk evaluation for the use of GTMPs in human subjects. Since the major beneficial therapeutic effects of the GTMP result from the contribution of both the delivery system of choice and transgene expression, it is expected that the study is designed to establish the following points⁷⁷:

- Pharmacodynamics in non-clinical model

The study should provide non-clinical evidence of the clinical benefit both *in vitro* and *in vivo* in a relevant animal model. The use of a validated predictive animal model to assess the clinical potential of the GTMP is preferred. Primary mechanism of action (MoA) should be addressed, and safety of the GTMP is of high importance (Box 2).

Box 2

Pharmacodynamics in non-clinical model for Pompe's disease

Methods and materials to assess the pharmacodynamics of HSCT in *Gaa*^{-/-} mouse and human cells are available, like mesenchymal stem cells and fibroblasts. The proposed GTMP should be titrated on these cells to evaluate the effect of the treatment and the treatment dose. These experiments can also contribute to a deeper understanding of the primary mechanism of action. The *Gaa*^{-/-} mouse model is an acceptable model to validate the effect of the GTMP. The mouse model mimics the disease in many ways; it has no residual *Gaa* activity which results in glycogen accumulation in virtually all tissues, cardiac myopathy, and progressive myopathy. Parameters that show therapeutic benefit in these animals involve: glycogen reduction in relevant tissues (like muscle and heart) and restoration (or preservation) of function (most importantly in skeletal muscle and heart). Safety issues regarding integrating vectors can be studied in the *Gaa*^{-/-} cells and mouse model. In hematopoietic stem cells, differentiation into all the hematopoietic lineages after transduction can be studied and pre-transplant integration profiles can be made. Transplanted animals should be followed lifelong and monitored for unwanted side effects of the treatment. At the end of the experiment, the transduced hematopoietic stem cells can be used for integration studies to assess the lentiviral integration pattern. Ideally, human GAA deficient HSCs should be transduced with the therapeutic vector and transplanted into an immunodeficient mouse. However, it is hardly possible to obtain enough HSCs from peripheral blood from a patient to execute these experiments.

- Bio-distribution data

Bio-distribution data should include details on all target and non-target organs, and address the persistence of the GTMP as well as the mobilization and shedding of the GTMP. The observation time should be long enough to obtain sufficient data of persistence of the GTMP. If possible, the dosing of the GTMP should translate to the desired dose in clinical use. The dose recommendation is a delicate point, since animal-to-human translation is of-

ten not easy. It should be based on the justification that the GTMP in the administered dose has a beneficial effect on the disease and that the initial biological effects in an appropriate animal model confirm the hypothesis of the study. When a dose recommendation is set, it will be refined by the results of the toxicity studies of the GTMP (Box 3).

Box 3

Bio-distribution data in Pompe's disease

To assess the bio-distribution of the GTMP in our proposed stem cell based gene therapy for Pompe's disease the transplanted mice should be monitored lifelong. Since the mechanism of action is excretion of GAA by the progenitors of the transduced HSCs and uptake by cells through the mannose-6-phosphate receptor and cross correction, all organs will be reached. This is exactly what is needed in Pompe's disease, since the accumulation of glycogen is in virtually every organ of the body. So all organs should be evaluated for glycogen content and GAA levels to assess the persistence of the incorporated gene. Since it is an *ex vivo* modification of the HSCs, mobilization and vector shedding experiments are not necessary. Dosing of the GTMP is not easy, as mentioned before. Mice-to-human translation is difficult to predict. However, an *in vitro* assay with human cells could predict in a more sophisticated way the needed GAA levels to obtain clearance in certain cell types. Furthermore, the animal experiments combined with the published data from Myozyme® (pre-clinical) experiments can be used to give a rough estimation of the GAA levels needed to obtain a decrease in glycogen accumulation. Toxicity studies need to be done in order to evaluate the possible effects of "overdosing". In Pompe's disease, we believe that there is not so much toxicity since the GAA is a lysosomal enzyme that does not reflect on regulatory pathways other than the breakdown of glycogen within the lysosomes. Toxicity in terms of too much of the viral vector on the HSCs in the transduction process is worth evaluating. In a clinical setting, the aim is to obtain maximal therapeutic benefit with as few copies of the GAA gene per cell as possible.

- Identification of potential organs targeted for toxicity and biological activity

Vector particle, structural components of the vector, integrations, and transgene expression all contribute to a potential toxicity of a GTMP. To study the effects *in vivo*, the GTMP should be administered through the same route and method of administration as in the proposed clinical protocol. To further assess toxicity and biological activity, the duration of treatment in the appropriate animal model should be long enough to observe potential toxicities and variance in response to the GTMP between the different animals sexes as these are important to consider in some cases, The toxicity of the GTMP should be assessed for the viral vector (complete expression vector including the cassette and the transgene), the number of transgene copies per genome, the possible effects of over-expression, and immunogenicity to the transgene. In some cases, re-administration of the GTMP may be needed; thus evaluation of immune response to the re-administered GTMP must be executed. Pharmacological side effects of the treatment should also be investigated. The

integration analysis is of high importance, the vector integration patterns and potential risks should be assessed, and oncogenic potential of GTMPs should be evaluated *in silico*. Also germline transmission of the vector should be tested for GTMPs as well as the virulence and replication of the GTMP. For some GTMPs, the animal models used are not representative of the human situation and therefore, might not provide satisfactory data⁷⁸.

In the case of modified cells, like HSCs, bio-distribution, migration of the cells, altered life span and differentiation abilities should be evaluated, as well as the possible immune reaction induced by the genetically modified cells. The environmental risk of the use of the GTMP should be assessed (Box 4).

Box 4

Identification of potential target organs of toxicity, biological activity in Pompe's disease

The method we propose for the development of a GTMP for Pompe's diseases involves a bone marrow transplantation with the patients own HSCs that are modified with an integrating lentiviral vector. Since it is not possible to perform autologous bone marrow transplantation in a mouse, we use an inbred strain of Gaa^{-/-} mice, what can be considered as an autologous transplantation. In our experiments, we have seen that only ~30% of the HSCs need to be genetically modified to obtain therapeutic effect. This implies that a full ablation of the bone marrow is not necessary: partial ablation should be sufficient. In the mouse model, we have achieved this by sub-lethal irradiation. In the patients, we consider other options, like a mild ablation that is chemically induced with Busulfan. This method can also be tested in the mouse model prior to clinical application if needed. In pre-clinical studies, the mice should be monitored lifelong to assess any side effects of the treatment as well as the efficacy over time. Regarding the toxicity of the vector, we need to evaluate the maximum copies per genome and whether the overexpression of the enzyme is toxic for the HSCs. Since the HSCs are modified, the immune cells will not consider the enzyme as foreign, and this opens possibilities in terms of additional administration of Myozyme® if needed, or a repeat of the bone marrow transplantation, without the risk of immunogenicity to the enzyme.

To determine the risks of insertional mutagenesis, vector integration patterns before and after transplantation on transduced HSCs should be evaluated for unwanted integration sites. Ideally, the HSCs should be screened for their integration patterns before transplantation into the patient. At this moment, due to difficulties in maintaining the stemness of the HSCs in culture and the time it takes to determine the integration profiles, this is not possible. Since *ex vivo* modification of the HSCs is executed, we do not need to assess the reactivation of the GTMP; after the overnight transduction, the HSCs are thoroughly washed before transplantation and no free vector is being administered.

Another aspect of the modified HSCs that needs to be evaluated is the migration pattern of the cells: do they migrate successfully to the bone marrow?, In vitro tests are also needed to evaluate whether these cells are still fully capable of differentiation into all the hematopoietic lineages. The environmental risk in the proposed GTMP is minimal since modified HSCs are transplanted. The patient is not exposed to the vector and the vector handling will be executed in a safe and controlled environment.

- The specific patients' eligibility criteria.

The study should provide insight which patient population has therapeutic benefit from the clinical trial and which patients will be included first. The patients' eligibility criteria should include a risk-benefit assumption for the specific treatment while taking the unmet medical need for these patients into consideration. (Box 5)

Box 5

The specific patients' eligibility criteria for Pompe's disease

There are different types of severity in Pompe's disease. Patients with the most severe classic-infantile phenotype are likely to benefit most from the stem cell based gene therapy. These patients are severely affected by the disease and show a clear response to ERT. Although these patients are weak, and bone marrow transplantation is an invasive treatment, we believe that these patients should be the first candidates in gene therapy trials. These very young children form a rather homogeneous group of patients in which survival can easily be set as clinical endpoints. Moreover, only a small fraction of the bone marrow needs to be ablated, so a minor conditioning regimen can be used. The effect of HSC based gene therapy will be far more difficult to assess in patients with non-classic forms of Pompe's disease since the patient population is far more heterogeneous and clinical end points are difficult to set. In addition, performing the HSC transplantation procedure is more risky in children and adults than in the very young infants leading to an unfavorable balance between the risk and the potential benefits these older patients may have. The non classic-infantile Pompe patients are a very heterogeneous population, with differences in residual GAA activity and a broad spectrum of disease manifestations. To include a homogenous group out of this heterogenous population might be more difficult. Inclusion criteria can involve for example a certain range of GAA activity measured in fibroblasts or muscle biopsies, or only patients that are or are not wheelchair or ventilator dependent. All depends on what the risk-benefit ratio is and this needs to be assessed per individual. In general, the more the disease has progressed, the more difficult it will be to completely reverse the skeletal muscle function, both in classic as in non classic-infantile patients.

ATMPs in the European Union and the Netherlands

Worldwide, at least 1902 gene therapy clinical trials have been approved from 1989 until 2012, with 408 in the EU (Figure 2). At the time of writing this manuscript, only 1 GTMP

has been registered in the EU,⁷⁹ Glybera®. It is important to identify the major stakeholders in the process of development and the reasons why it appears to be difficult to get GTMPs to patients beyond a clinical setting.

GTMP clinical trials in the Netherlands

Until now, 32 clinical trials with GTMPs have been or are currently being conducted in the Netherlands (Figure 9)⁸⁰⁻⁸³. The first trial started in 1991 and involved a stem cell based retroviral gene therapy for patients with adenosine deaminase (ADA) deficiency and was initiated by Prof. dr. D. Valerio^{84,85}. The two latest trials started in April 2012 and June 2012. One involved a multicenter clinical trial to determine whether autologous T-cells modified with a retroviral vector can be of benefit in advanced stage melanoma patients⁸⁶. The other clinical trial initiated in June 2012 investigates the response to infusion of retroviral transduced T-cells to complement allogeneic stem cell transplantation in patients with a high risk of developing leukemia⁸⁷.

Sponsors and stakeholders in the development of GTMPs

The majority of stakeholders in the development of ATMPs are academia, small and medium size enterprises (SMEs), charities, and other small companies (Box 6)⁸⁸. In general, these stakeholders have limited financial resources for the development and licensing of an ATMP and limited-to-no regulatory expertise. Limited financial resources are a showstopper in the developmental process, wasting many years of conducted research. In some cases, when the development of the ATMP is in the advanced strata of the licensing process, the initial SME runs out of funding. The licensing of the ATMP then either needs to be put on hold, or it needs to be refinanced, as was the case in the development of Glybera®.

Box 6

Stakeholders for Pompe's disease

The development of the proposed GTMP for the treatment of Pompe's disease so far has been entirely done in academic setting with no involvement of any pharmaceutical company or other SME. To proceed to the next step, financial resources need to be expanded, and preferably by joining forces with a SME or larger pharmaceutical company to ensure financial support up to market authorization. Besides involvement of an SME or large pharmaceutical company, the regulatory offices should also be involved at an early stage of development to speed up the process of marketing approval.

In the case of the development of GTMPs, academic institutions or small companies are the major sponsors, and some charities are involved. Academic institutions are almost absent in the manufacturing process of GTMPs, which require a highly specialized infrastructure

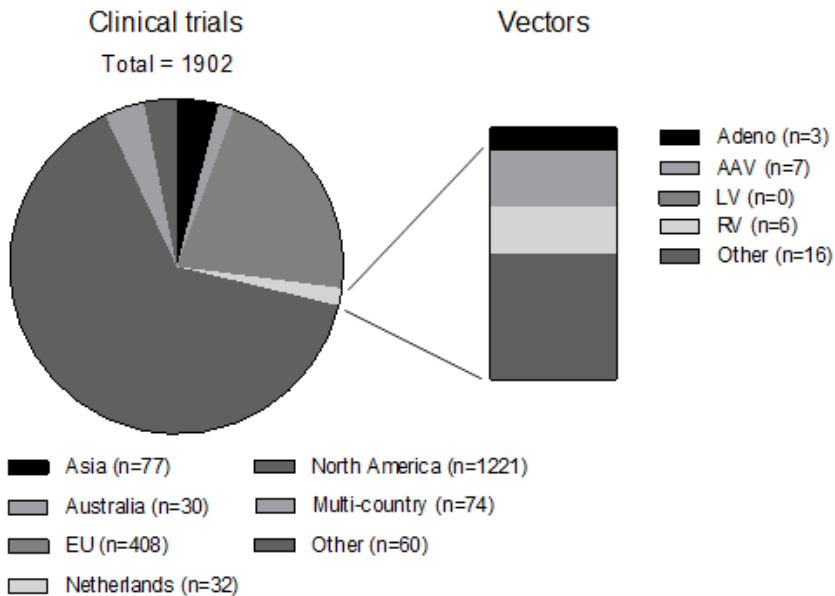


Figure 8: Distribution of gene therapy clinical trials and vectors. On the right, vectors used in the Netherlands are specified in detail (based on *The Journal of gene medicine*, 2013, John Wiley and Sons Ltd.).

and high costs are involved⁸⁸. The authors believe that large pharmaceutical companies are absent in the developmental process in the majority of cases, due to the uncertainties with respect to the regulatory process. Usually they become involved once a product is in an advanced state and preclinical data are promising; however, large companies such as GlaxoSmithKline and Novartis have become more and more involved in gene therapy projects in the past years.

Why is only one GTMP approved so far and what are the lessons learned from it

The question still remains why Glybera is the first GTMP ever approved in the Western World. In short, AMT had a candidate product for a rare disease, had the right technology, and was well financed at the start. This is not to say that other companies with similar products that did not make it to final market authorization were in one way or another less equipped than AMT. It seems that continuous pressure from the AMT on the regulatory systems demanded that the regulators treated this GTMP as an exceptional product for a very rare and serious disease. Despite a steep learning curve for both researchers and regulators, the first GTMP was ultimately approved.

The process of licensing Glybera has been transparent enough to draw some concluding lessons from this case⁷³. The essentials are to have a good clinical practice throughout the entire process, get the data straight, carefully consider the endpoints of the trial, review

the patient population thoroughly, and to include the regulatory agencies in the early stages of the process.

Future outlook

The first approval of a gene therapy medicinal product is a boost for the gene therapy field. After negative headlines in the late 90s, gene therapy had become almost science fiction and the general public seems to have lost confidence that gene therapy could be the cure for many (inherited) diseases⁸⁹. Fortunately, most labs involved in development of gene therapy products diligently worked to overcome technical problems and by producing solid science, overcoming the prejudices facing gene therapy. Finally, they have succeeded.

The approval of Glybera brings forth a renewed, yet cautious belief in the potential of gene therapy and the implications for the advancement of therapeutics. Potential GTMPs that are likely to achieve marketing authorization involve, for example, GTMPs for the treatment of ADA-SCID, Leber congenital amaurosis, and Hemophilia B⁹⁰⁻⁹². Developments in the field of gene therapy are happening so rapidly that the current traditional road to medicinal licensing is inappropriate to provide access to these promising new drugs for patients with unmet medical needs. For a GTMP, by the time it reaches the market, it is already outdated by newer scientific developments. Although companies are encouraged to seek scientific advice from regulatory agencies, it appears that the current financial and regulatory hurdles are too high for most companies to succeed in product licensing. It will take courage and political will to open this issue up for discussion. With a more applicable approach of trial approval and regulating patient access, gene therapy, as the ultimate form of personalized medicine, might fulfill its promise soon (Box 7).

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Box 7

Future for stem cell based lentiviral gene therapy for Pompe's disease

Results obtained from the experiments described in this thesis show the promise of a new therapy for Pompe's disease. However, as can be learned from the lessons in the Glybera case, and the conditional necessities described in the preceding boxes, many steps have to be taken before this GTMP is ready for clinical trial and especially market authorization, but we believe gene therapy is the future for patients with Pompe's disease and many other monogenic disorders.

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Stem cell based gene therapy

for Pompe's disease

acid alpha-glucosidase

Integrations

Hematopoietic stem cells

Muscle

Mouse

Single intervention

Lentivirus

Future

Storage

ERT

Transplantation

Costs

Glycogen

Immune tolerance

Transplantation

SUMMARY

Pompe's disease is a so-called "lysosomal storage disorder" whereby an endogenous substrate accumulates in a cellular compartment due to an inherited genetic defect. The gene affected in Pompe's disease is the acid alpha-glucosidase gene (abbreviated as *GAA*) that encodes the synthesis of an enzyme with the same name that is responsible for the breakdown of lysosomal glycogen into glucose. As a result of the enzyme deficiency in Pompe's disease, glycogen accumulates in virtually all cells of the body and causes damage to organs and eventually loss of function. Skeletal muscle is primarily affected and muscle weakness is a prominent symptom of Pompe's disease.

The clinical course of Pompe's disease is highly variable, depending on the severity of the enzyme deficiency. Two major types of Pompe's disease are distinguished. The most severe classic-infantile form is characterized by profound cardiomyopathy and severe muscle weakness. The first symptoms manifest early after birth and most patients succumb in their first year of life due to cardio-respiratory failure. Less severe forms with usually later onset are characterized by skeletal muscle weakness and respiratory problems while cardiac involvement is not evident. Most of these latter patients become wheelchair dependent and might need respiratory support. The current treatment available for Pompe's disease is enzyme replacement therapy (ERT). ERT involves the intravenous infusion of recombinant human acid alpha-glucosidase (alglucosidase alfa) as a means to replace the missing endogenous enzyme and so to degrade the lysosomal glycogen.

In **chapter 1** Pompe's disease is introduced, discusses the pros and cons of ERT and the application of gene therapy as an alternative therapeutic approach. The studies described in this thesis are focused at the development of hematopoietic stem cell mediated gene therapy using lentiviral vectors as a transfer vehicle. An important feature of stem cells is that they can replicate themselves and differentiate into various cell lineages. Hematopoietic stem cells home to the bone marrow, self-renew and continuously supply the blood with new red and white blood cells and blood platelets. Thus, transplanted genetically modified hematopoietic stem cells secure lifelong the production of genetically modified blood cells. Stem cell gene therapy in Pompe's disease aims at production and secretion of acid alpha-glucosidase by blood cells that clears the lysosomal glycogen in other tissues by cross-correction. The transplanted cells are derived from the patient and will not be recognized as foreign by the patients' immune system. By the same token, also the enzyme produced by these cells will likely not be recognized as a foreign compound, as immune tolerance is induced by lymphoid descendants of the genetically modified stem cells.

The phenomenon of immune tolerance is addressed in **chapter 2** in which a mouse model of Pompe's disease is used to investigate the potential of stem cell based gene therapy. These

mice have, just as infants with Pompe's disease, weak skeletal muscles and cardiomegaly due to a total deficiency of acid alpha-glucosidase. Hematopoietic stem cells of these mice were isolated and *ex vivo* genetically modified with lentiviral vectors that contain a functional *GAA* gene construct. In these studies we used the strong "spleen focus forming virus promoter" to drive gene expression. The major finding in these series of experiments was the correction of the cardiac abnormalities, since infants with Pompe's disease succumb from cardio-respiratory failure. Reduction of the glycogen content was observed in several tissues but least so in skeletal muscle, the major target organ in Pompe's disease. However, the outcomes of functional tests were suggestive for a slight improvement of skeletal muscle and pulmonary function. Furthermore, the transplanted mice appeared to be immune tolerant for recombinant human *GAA* when administered intravenously.

In **chapter 3** the same strategy was used as in Chapter 2 to investigate whether mice with Pompe's disease could be rescued by hematopoietic stem cell mediated gene therapy, with the difference that a codon-optimized *GAA* gene construct (*GAAco*) was used. Codon-optimization is a generally accepted methodology and mostly leads to higher gene expression. In this chapter it is shown that codon-optimization indeed leads to a much higher expression of the transgene in hematopoietic stem cells and their blood cell derivatives. As a result hardly any glycogen storage could be detected in the organs that were analyzed, including skeletal muscle, at 10 months after the mice were transplanted with the *GAAco* construct. Notably, the glycogen storage in brain cells was also reduced. Clearance of glycogen was reflected in improved muscle function; the treated mice performed equally well as healthy mice in voluntary running wheels.

One of the features of lentiviral vectors is that they integrate fairly random in the genome. They may integrate near proto-oncogenes and tumor suppressor genes and may interfere with the function of these genes. Thus, it is important to check the integration sites. In our studies we have not seen any integration-related adverse events in the transplanted animals. However, more extensive studies should be conducted.

To minimize the risk of insertional mutagenesis we have explored in **chapter 4** the efficacy of hematopoietic stem cell mediated gene therapy aiming at approximately one integration per genome. This strategy was evaluated in young mice without detectable glycogen storage, and in old mice with advanced glycogen accumulation. The outcome of this experiment was that one integration per genome is insufficient to correct the lysosomal glycogen storage in mice with Pompe's disease.

Chapter 5 is a review addressing another type of stem cells, namely muscle stem cells, and their potential use for (gene) therapy. The use of muscle stem cells in Pompe's disease is

appealing since muscle damage is the main pathologic feature. With the present state of knowledge, the application of these cells for therapeutic purposes is still limited because they are difficult to propagate while maintaining their stem cell characteristics.

The discussion, **chapter 6**, consists of two parts. The first part is a general discussion about therapy in Pompe's disease and the development of hematopoietic stem cell mediated gene therapy as described in the other chapters of this thesis. The second part outlines the regulatory road to market authorization for advanced therapeutic medicinal products and in particular gene therapy medicinal products. Recently, the first gene therapy products obtained market approval. The regulatory procedures that were used for the approval of Glybera, the first gene therapeutic product that obtained market approval, were described in detail by the European Medicine Agency (EMA). This has clarified the procedures and is expected to facilitate the approval of gene therapeutic products for other diseases.

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The studies described in this thesis have provided insight in the potency of hematopoietic stem cell mediated gene therapy for the treatment of Pompe's disease. Several important aspects require further attention; above all finding the optimal balance between the number of integrations and the level of acid alpha-glucosidase expression to optimize therapeutic benefit and minimize risk.

NEDERLANDSE SAMENVATTING

De ziekte van Pompe is een zogeheten 'lysosomale stapelingsziekte' en het gevolg van een genetisch defect waardoor een bepaalde lichaamseigen stof zich ophoopt in een celcompartiment (organel) dat lysosoom genoemd wordt. Het deficiënte gen is het zure alfa-glucosidase gen en bevat informatie voor de synthese van het enzym zure alfa-glucosidase (GAA) dat lysosomaal glycogeen afbreekt tot glucose. Bij de ziekte van Pompe wordt er door een fout in dit gen geen of niet goed functionerend enzym geproduceerd en hoopt het glycogeen zich op in de lysosomen. De glycogeenstapeling veroorzaakt schade aan de cel en kan uiteindelijk een geheel orgaan beschadigen, hetgeen tot disfunctioneren van dit orgaan kan leiden. Hoewel de glycogeenstapeling in alle cellen van het lichaam aanwezig is, zijn de spieren het meest aangedaan bij patiënten met de ziekte van Pompe. Twee hoofdvormen van de ziekte van Pompe worden onderscheiden. De meest ernstige vorm, waarbij naast de skeletspieren ook het hart is aangedaan, wordt de klassiek infantiele vorm genoemd. Alle andere vormen waarbij het hart niet of slechts in heel lichte mate is aangedaan worden collectief aangeduid als niet-klassieke vormen. De klassiek infantiele vorm van de ziekte van Pompe manifesteert zich vrijwel meteen na geboorte en de gemiddelde leeftijd waarop deze patientjes komen te overlijden is 6 tot 8 maanden. Zij overlijden in de meeste gevallen aan hart- en/of ademhalingsfalen, dit laatste vanwege zwakte van de ademhalingsspieren. Ziekte verschijnselen bij patiënten met de niet-klassieke vorm van de ziekte van Pompe zijn skeletspierzwakte en ademhalingsproblemen; die zich op elke leeftijd voor het eerst kunnen manifesteren.

Vrijwel alle patiënten worden rolstoel-afhankelijk en hebben uiteindelijk ondersteuning van de ademhaling nodig. De huidige therapie voor patiënten met de ziekte van Pompe is enzym vervangingstherapie (ERT). Bij deze vorm van therapie krijgen de patiënten elke week of om de week recombinant humaan zure alfa-glucosidase per infuus toegediend om zo het gestapelde glycogeen af te breken.

In **hoofdstuk 1** worden de voor- en nadelen besproken van ERT voor de ziekte van Pompe. ERT lost de hartkwalen van kinderen met de klassiek infantiele vorm van de ziekte van Pompe vrijwel volledig op, vertraagt het ziekte beloop en verlengt hun levensverwachting aanzienlijk. Het merendeel van de volwassen patiënten heeft ook baat bij ERT. Een nadeel van ERT is dat de therapie minimaal eens in de twee weken en levenslang moet worden toegepast. Sommige patiënten ontwikkelen een dusdanige hoeveelheid antilichamen tegen het toegediende enzym dat de effectiviteit van ERT vermindert. Bovendien zijn de kosten van de behandeling hoog. Er wordt daarom gezocht naar een beter alternatief.

Dit proefschrift gaat over het ontwikkelen van een alternatief voor ERT voor de ziekte van Pompe, namelijk "gentherapie", gebaseerd op transplantatie van genetisch ge-

modificeerde hematopoietische stamcellen. Eén van de belangrijkste eigenschappen van stamcellen is dat deze cellen in staat zijn zichzelf te vermeerderen en te ontwikkelen tot cellen met specifieke functies. Hematopoietische stamcellen nestelen zich na intraveneuze transplantatie in het beenmerg en zorgen voor de aanmaak van rode en witte bloedcellen en bloedplaatjes. Dus, als er genetisch gemodificeerde hematopoietische stamcellen worden getransplanteerd kunnen deze cellen in principe levenslang voor de aanmaak van genetisch gemodificeerde bloedcellen zorgen. Bij de aanpak van de ziekte van Pompe is het doel de eigen hematopoietische stamcellen van een patiënt dusdanig genetisch te veranderen dat de daaruit voortkomende bloedcellen grote hoeveelheden zure alfa-glucosidase gaan produceren en uitscheiden. Het uitgescheiden enzym kan via de bloedbaan door andere cellen opgenomen worden. Omdat de genetisch gemodificeerde stamcellen bij deze procedure van de patiënt zelf afkomstig zijn zullen er geen afstotingsverschijnselen ontstaan ten gevolge van het transplantaat. De cellen zullen door het immuunsysteem als "eigen" worden beschouwd. Ook het enzym zal waarschijnlijk door de cellen als eigen herkend worden als gevolg van immuun tolerantie geïnduceerd door de lymfocyttaire afstammelingen van de genetische gemodificeerde stamcellen.

In hoofdstuk 2 wordt het verschijnsel immuun tolerantie uitvoerig beschreven in een muismodel voor de ziekte van Pompe. Het muismodel dat gebruikt wordt voor deze studies heeft totaal geen zure alfa-glucosidase en heeft net als kinderen met de klassiek infantiele vorm van de ziekte van Pompe een vergroot hart, slappe spieren en ademhalingsproblemen. In **hoofdstuk 2** wordt aan de hand van dit muismodel met succes het principe van een transplantatie met gemodificeerde hematopoietische stamcellen aangetoond. De stamcellen worden gemodificeerd met behulp van lentivirale vectoren. Deze vectoren zijn afgeleid van virussen die ergens in het menselijke DNA kunnen integreren en aldus een gen introduceren. Om een goede expressie van dat gen te bewerkstelligen is het noodzakelijk dat het gen wordt aangedreven door een sterke "promoter". Er zijn verschillende promotoren die geschikt zijn; een ervan is de "spleen focus forming virus (SFFV) promoter". Deze virale promoter staat bekend om de hoge mate van genexpressie. Wij hebben deze promoter in combinatie met het humane zure alfa-glucosidase gen gebruikt om dit gen in hematopoietische stamcellen van de muis te brengen. Dit doen we door de stamcellen van de muis te isoleren en buiten het lichaam te veranderen met de lentivirale vectoren alvorens deze in andere muizen met de ziekte van Pompe te transplanteren. Om de gemodificeerde hematopoietische stamcellen weer efficiënt in het beenmerg terug te plaatsen is het noodzakelijk daarin ruimte te maken door een deel van de bestaande hematopoietische stamcelpopulatie te verwijderen. Om dit te bewerkstelligen worden de muizen voorbehandeld met gamma-straling. Na transplantatie is ongeveer 30% van de totale hematopoietische stamcelpopulatie genetisch gemodificeerd en wordt een significante hoeveelheid zure alfa-glucosidase geproduceerd door de witte bloedcellen. De

bloedcellen scheiden het enzym uit dat vervolgens opgenomen wordt door andere cellen, zoals spier-, hart-, lever en miltcellen. In veel van deze weefsels wordt een flinke afname van de hoeveelheid glycogeen waargenomen, maar in skeletspieren zoals de quadriceps femoris (bovenbeenspier) blijft veel glycogeenstapeling aanwezig. Dit blijkt ook uit testen met betrekking tot skeletspier-functie; er is een lichte verbetering te meten ten opzichte van onbehandelde Pompe muizen, maar de behandelde muizen functioneren nog niet zo goed als de gezonde muizen. Wel zorgt de gentherapie ervoor dat het hart normaal functioneert. Dit kan gezien worden als een belangrijke mijlpaal aangezien hartfalen een belangrijke doodsoorzaak is bij de klassiek infantiele vorm van de ziekte van Pompe. Ook ademhalingstesten laten een verbetering bij behandelde muizen zien.

In **hoofdstuk 3** wordt een aangepast humaan zure alfa-glucosidase gen construct gebruikt (*GAAco* ipv *GAA*), met behulp van algoritmes werd de code van het gen geoptimaliseerd om maximale expressie te krijgen. Dit hoofdstuk laat zien dat met het *GAAco* construct inderdaad enorm hoge expressie-niveaus bereikt kunnen worden, zo hoog, dat vrijwel alle glycogeenstapeling verdwijnt. De muizen die met het *GAAco* construct behandeld werden, op dezelfde manier als in hoofdstuk 2, zijn gevolgd gedurende 10 maanden, waarna allerlei weefsels geanalyseerd werden en functionele testen werden uitgevoerd. De behandelde muizen lopen net zo hard en zo veel in loopwielen als gezonde muizen en dit valt te verklaren uit het feit dat er vrijwel geen glycogeenstapeling meer meetbaar was na toepassing van gentherapie. Dat de spierfunctie normaliseert bij deze wijze van gentherapie is een belangrijk gegeven omdat volledige correctie van spierfunctie bij toepassing van ERT een grote uitdaging blijft. Als bijzonder resultaat laat dit hoofdstuk zien dat heel hoge expressie van zure alfa-glucosidase ook tot gedeeltelijke correctie van glycogeenstapeling in de hersenen leidt, doordat een deel van de microglia cellen in het brein het *GAAco* gen construct tot expressie brengen. Vanwege het gebruik van lentivirale vectoren, die zich in het DNA van de hematopoietische stamcel nestelen, is het van belang om te controleren of er door de "integratie" geen schadelijke veranderingen in het DNA zijn opgetreden. Ook geldt dat, hoe minder integraties van het therapeutische gen er nodig zijn om voldoende zure alfa-glucosidase te produceren om het glycogeen af te breken, des te kleiner de kans is op schadelijke effecten door integraties. In hoofdstuk 3 wordt een eerste schatting gemaakt van het aantal integraties rond proto-oncogenen en tumor-suppressor genen. Dit zijn genen die een rol spelen bij het ontstaan van kanker. Tot op heden hebben wij bij muizen geen integratie-gerelateerde bijwerkingen gezien. Echter, om de kans op integratie-gerelateerde bijwerkingen zo klein mogelijk te houden, is het zaak om met zo min mogelijk integraties een zo groot mogelijk therapeutisch effect te bereiken.

In **hoofdstuk 4** wordt het therapeutisch effect bij een gemiddelde integratie van 1 viraal kopie per genoom in het Pompe muis model beschreven. In dit experiment werden jonge

muizen behandeld waarbij nog geen glycogeenstapeling detecteerbaar was en oudere muizen met vergevorderde glycogeenstapeling. In dit experiment bleek één integratie per genoom niet voldoende voor de effectieve behandeling van de oudere noch de jongere muizen. Deze experimenten dienen nog herhaald en uitgebreid te worden om een definitieve uitspraak te kunnen doen over het aantal benodigde integraties en de veiligheid van de procedure.

Hoofdstuk 5 is een uitgebreid overzicht van verschillende soorten spierstamcellen die eventueel gebruikt kunnen worden voor gentherapie. De toepassing van spierregenererende cellen is aantrekkelijk omdat bij spierziekten, zoals de ziekte van Pompe, spierschade een prominent verschijnsel is. Een nadeel van deze cellen is dat ze lastig te hanteren zijn en moeilijk vermeerderd kunnen worden buiten het lichaam, hetgeen de toepassing ervan op dit moment limiteert. Er gaat wereldwijd veel aandacht uit naar het verbeteren van kweekcondities van spier-stamcellen.

Hoofdstuk 6 bestaat uit twee delen. Een meer algemene discussie over therapie bij de ziekte van Pompe en de ontwikkeling van hematopoietische stamcel gemedieerde gentherapie, zoals beschreven in de diverse hoofdstukken van dit proefschrift. Daarnaast een deel over regelgeving omtrent de toepassing van genterapeutische producten en de weg naar markttoelating. Nadat een therapie, zoals in dit proefschrift beschreven, uitvoerig getest is in verschillende (dier)modellen, kan deze in patiënten getest worden. Wanneer de resultaten van klinisch onderzoek overtuigend zijn, kan de stap naar markttoelating gemaakt worden. Het middel of de procedure moet dan eerst goedgekeurd worden door de betrokken instanties zoals de European Medicines Agency (EMA). Recent hebben in Europa de eerste gentherapie producten een toelating tot de markt gekregen. In het laatste deel van de discussie wordt de gang van zaken omtrent markttoelating in kaart gebracht, met de ziekte van Pompe en de experimenten beschreven in dit proefschrift als voorbeeld. De dossierbeoordeling van genterapeutische producten is lange tijd een "black box" geweest, mede doordat de regelgeving niet transparant werd toegepast. De beoordeling van het eerste toegelaten genterapeuticum Glybera is door de EMA in detail beschreven. Daardoor is het nu duidelijker hoe de beoordeling in zijn werk gaat en zal het waarschijnlijk minder tijd kosten om tot markttoelating voor andere genterapeutische producten te komen.

De studies beschreven in dit proefschrift hebben veel inzicht gegeven in de toepassing van hematopoietische stamcel gemedieerde gentherapie voor de ziekte van Pompe. In het muismodel werd aangetoond dat deze benadering in principe kan leiden tot vrijwel volledige correctie van het ziektebeeld. Daarmee is de belangrijke basis voor toepassing bij patiënten gelegd. Verscheidene belangrijke aspecten dienen nog wel aandacht gegeven te

worden, in het bijzonder het vinden van de optimale balans tussen het aantal integraties van de virale vector in de stamcellen en effectieve zure alfa-glucosidase productie. Klinisch onderzoek bij patiënten zal uiteindelijk uitsluitel moeten geven over de effectiviteit en veiligheid van deze therapie als alternatief voor de huidige enzymvervangings therapie.

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ABBREVIATIONS

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AAV	Adeno-associated virus
ADA	Adenosine deaminase
ALD	Adrenoleukodystrophy
AMT	Amsterdam Molecular Therapeutics
APC	Antigen-presenting cells
ARSA	Arylsulfatase A
ATMP	Advanced-therapy medicinal product
BCA	Bicinchoninc acid
BGGO	Bureau Genetisch Gemodificeerde Organismen
BM	Bone marrow
BMDC	Bone marrow derived cells
BMT	Bone marrow transplantation
CAT	Committee for Advanced Therapies
CBG	College ter Beoordeling van Geneesmiddelen
CCMO	Centrale Commissie Mensgebonden Onderzoek
CGD	Chronic granulomatous disease
CHMP	Committee for Medicinal Products for Human Use
CHO	Chinese hamster ovary
CMV	Cytomegalovirus
CNS	Central nervous system
COGEM	Commissie Genetische Modificatie
COPD	Chronic Obstructive Pulmonary Disease
CRIM	Cross-reactive immunological material
CTMP	Cell therapy medicinal product
DAPI	4',6-diamidino-2-phenylindole
diaph	Diaphragm
DMD	Duchenne muscular dystrophy
DNA	Deoxyribonucleic acid
EDL	Extensor digitorum longus
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
eMHC	Embryonic myosin heavy chain
ER	Endoplasmic reticulum
ERT	Enzyme replacement therapy
ES	Embryonic stem cells
EU	European Union
EURORDIS	Rare diseases Europe

FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
FSHD	Facioscapulohumeral muscular dystrophy
FVB/N	Friend leukemia virus B, sensitive to the N strain
G-CSF	Granulocyte colony-stimulating factor
GAA	Human acid alpha-glucosidase
Gaa	Mouse acid alpha-glucosidase
GAAco	Human acid alpha-glucosidase codon-optimized
GALC	Galactosylceramidase
Gastr	Gastrocnemius
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GILT	Glycosylation- independent lysosomal targeting
GMA	Glycol methanacrylate
GSD	Glycogen storage disorder
GT	Gene therapy
GTMP	Gene therapy medicinal product
GYG	Glycogenin
GYS	Glycogen synthase
HE	Hematoxylin and Eosin
hESC	Human embryonic stem cell
HIV-1	Human immunodeficiency virus
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HSPC	Hematopoietic stem and progenitor cells
IenM	Ministerie van Infrastructuur en Milieu
IGF	Insulin-like growth factor
IL2RG	Interleukin 2 Receptor gamma chain
iPS	Induced pluripotent stem cells
KO	Knock-out
LAM-PCR	Linear amplification-mediated polymerase chain reaction
Lin-	Lineage negative
LPLD	Lipoprotein lipase deficiency
LSD	Lysosomal storage disorder
LTR	Long terminal repeat
LV	Lentiviral vector
LVMI	Left ventricular mass index

M6P	Mannose-6-phosphate
MDX	Muscular dystrophin deficient
miRNA	Micro ribonucleic acid
MLD	Metachromatic leukodystrophy
MoA	Mechanism of action
MOI	Multiplicity of infection
MPS	Mucopolysaccharidosis
MRF	Muscle regulatory factor
mRNA	Messenger ribonucleic acid
MTT	Myoblast transfer therapy
MuSC	Muscle stem cells
ND	Not done
NOD/SCID	Nonobese Diabetic/Severe combined immunodeficiency
ORF	Open reading frame
PAS	Periodic acid Schiff
Pax	Paired box
PB	Peripheral blood
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Pegylation
PFA	Paraformaldehyde
PGK	Phosphoglycerate kinase
PIF	Peak inspiratory flow
PNS	Peripheral nervous system
PPT	Polypurine tract
QF	Quadriceps femoris
Quad	Quadriceps femoris
RC	Reserve cell
Rev	Responsive element
RRE	Rev responsive element
SC	Stem cell
SCF	Stem cell factor
SEM	Standard error of mean
SF(FV)	Speen focus-forming virus
SIN	Self inactivating
SME	Small to medium enterprise
SPC	Summary of product characteristics
SRT	Substrate reduction therapy
SuC	Supportive care

T-ALL	T-lineage acute lymphoblastic leukemia
TA	Tibialis anterior
TALEN	Transcription activator-like effector nuclease
TEP	Tissue engineering product
TV	Tidal volume
UCB	Umbilical cord blood
UCOE	Ubiquitous chromatin opening element
VCN	Vector copy number
VSV-G	Vesicular stomatitis virus G protein
VWS	Volksgesondheid, welzijn en sport
Wks	Weeks
WPRE	Woodchuck hepatitis virus posttranscriptional regulatory element
WT	Wild type
X-SCID	X-linked severe combined immunodeficiency

PHD PORTFOLIO

Name PhD student: Merel Stok
Erasmus MC Department: Hematology
Research School: MolMed

PhD period: September 2008- February 2013
Promotors: Prof. dr. G. Wagemaker, Prof. dr. A.G. Vulto
Supervisors: Dr. A.J.J. Reuser, Dr. N.P. van Til

1. PhD training

	Year	Workload (Hours/ECTS)
General academic skills		
- Workshop Photoshop and Illustrator	2011	0.3
- Biomedical English Writing and Communication	2010	2.0
- Laboratory animal science (art. 9)	2008	3.0
Research skills		
- Basic course on SPSS	2010	0.4
- The Course Molecular Medicine	2007	0.6
In-depth courses (e.g. Research school, Medical Training)		
- Stanford continuing studies: From idea to business plan	2012	0.4
Presentations		
- Molecular Medicine Day 2013, (oral presentation), Rotterdam, the Netherlands	2013	1.0
- Pompe expert day (oral presentation), Rotterdam, the Netherlands	2010	1.0
- 2 x Nederlandse Vereniging voor Gen en Cel therapie (oral presentations), Heeze, the Netherlands	2009, 2010	2.0
International conferences		
- Drug Information Association, Euromeeting (oral presentation), Amsterdam, the Netherlands	2013	0.2
- 2 x American Society for Gene and Cell Therapy, Boston (poster presentation, 2008), Salt Lake City (oral presentation, 2013), USA	2008, 2013	2.0
- 2 x European Society for Gene and Cell Therapy, Rotterdam, the Netherlands (oral presentation) and Birmingham, UK (poster presentation)	2007, 2011	2.0
- World Muscle Society (poster presentation), Albufeira, Portugal	2011	1.0
- ASGD-UK Patient and Family Conference (oral presentation), Telford, UK	2011	1.0
- 4 x CONSERT / PERSIST annual meeting (3 oral presentations, 1 poster presentation), Leukerbad, Switzerland	2008-2011	4.0
- European Society for Human Genetics (poster presentation), Amsterdam, the Netherlands	2011	1.0
- Steps Forward in Pompe Disease, annual meeting (poster presentation), Munich, Germany	2009	1.0
Seminars and workshops		
- British Society of Gene and Cell Therapy, London, UK	2012	0.2
- Acid Maltase Deficiency Association Meeting, San Antonio, USA	2011	0.4
- Pompe expert day, Rotterdam, the Netherlands	2009	0.4
2. Teaching activities		
Lecturing		
- 3 x Lecturing 2 nd year elective course on gene therapy for medical students	2009-2012	0.1
Supervising practicals and excursions		
- 2 x practicals 2 nd year elective course on gene therapy for medical students	2010, 2012	0.1
Supervising		
- 2 x Bachelor student (20 weeks)	2010-2012	3.0
- co-supervising Master and MLO student	2010	1.5

GRANTS AND AWARDS

Grants/ fellowship:

Training fellowship from the EU project “CONCERT” for 9 months, 2007 (Lentiviral gene therapy for MPSI, Milan, Italy)

NWO TopTalent grant, 2008-2012 (funding for 4-years PhD: Development of an ex vivo lentiviral gene therapy approach based on hematopoietic stem cells for treatment of Pompe’s disease)

Sophia Foundation Grant 2011-2013 (Hematopoietic stem cell gene therapy for MNGIE)

Awards:

Poster Award in Basic Science; 3rd European Symposium Steps Forward in Pompe’s disease 2009, Munich, Germany

Leslie Fairbairn award for oral presentation 2011, Leukerbad, Switzerland
SEBM 2011 **International prize** at European Society for Cell and Gene Therapy, Brighton, United Kingdom

Molecular Medicine day 2013, Award for best oral presentation, Rotterdam, the Netherlands

Travel Grant, American Society of Gene and Cell Therapy 2013, Salt Lake City, United States of America

CURRICULUM VITAE

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Merel Stok werd geboren 25 oktober 1983 te Rotterdam. Zij volgde het voorbereidend wetenschappelijk onderwijs (VWO) aan de Openbare Scholengemeenschap De Ring van Putten te Spijkenisse van 1996 tot 2002. Hierna startte zij de opleiding Biomedische Wetenschappen aan de Universiteit van Leiden om vervolgens het Hoger Laboratorium Onderwijs te Rotterdam volgen. Na behalen van het diploma in 2006 volgde zij de Master of Molecular Medicine aan de Erasmus Universiteit Rotterdam, waaronder keuzeonderzoek op de afdeling Hematologie van het Erasmus Medisch Centrum te Rotterdam onder supervisie van Prof. dr. G. Wagemaker. Deze heeft tevens het afstudeeronderzoek aan de San Raffaele Universiteit in het laboratorium van Prof. dr. L. Naldini te Milaan, Italië gesuperviseerd in 2007-2008. Hiervoor ontving zij een exchange fellowship van het Europese samenwerkingsproject CONSERT. Na afstuderen startte zij het promotieonderzoek onder leiding van Prof. dr. G. Wagemaker, gesteund door een NWO TopTalent beurs voor de volledige PhD periode. Het promotieonderzoek resulteerde in het proefschrift dat nu voor u ligt. In 2012 heeft zij een half jaar ervaring op het terrein van spierbiologie opgedaan in het lab van Prof. T. Rando, PhD., Stanford University, USA en is per 1 april 2013 werkzaam als wetenschappelijk onderzoeker bij het Centrum voor Lysosomale en Metabole Ziekten in het Erasmus MC te Rotterdam, onder leiding van Prof. dr. Ans T. van der Ploeg en Dr. Pim W.M. Pijnappel.

DANKWOORD

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Allereerst wil ik mijn dankbaarheid uiten aan mijn promotor Professor Gerard Wagemaker. Gerard, ik kan je niet genoeg bedanken voor alle mogelijkheden die je mij hebt geboden. Wanneer ik weer eens voor je op en neer aan het springen was vanwege nieuwe ideeën voor het Pompe onderzoek, draaide je je met een "hallo Merel" in je stoel om en luisterde je naar de nieuwe plannen. Niet dat die altijd geweldig en baanbrekend waren, wat je mij dan ook haarfijn uitlegde, maar ik heb het altijd als erg prettig ervaren. Voor de vrijheid die je mij hebt gegeven ben ik je ontzettend dankbaar, net als de mogelijkheden om mij te ontplooiën als wetenschapper. Door jou heb ik in het lab van Luigi Naldini een enorm leerzame tijd gehad, en ook toen er zich een mogelijkheid voordeed naar Stanford te gaan heb je mij gesteund. Ondanks alle perikelen rondom jouw pensioen, ligt er toch een mooi proefschrift! "*C'est la vie*" zoals je zegt. Gerard, ik heb een goede tijd bij jou gehad, bedankt.

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Then there is my lab-buddy: Marshall!!! We have been through the PhD-process together and became friends. And what could be better than having our PhD ceremony on the same day?! I miss our daily chats, conferences all over the world and your relaxed way of looking at things. We have a great time to look back at. Fortunately, you have got a new job, being group-leader, postdoc and technician all in 1. I am sure you can do it, I wish you all the best together with Noemi and remember, we can always: "change, into a truck".

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